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Time course of ethanol evolution during the early germination of artificially aged soybean seeds

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by

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ABSTRACT

Metabolic production of ethanol in seeds increases with loss of vigor. The objectives of this study were: (1) to compare ethanol evolution patterns in imbibing soybean seeds which were artificially aged using two methods to produce different levels of vigor, (2) to examine the relationship between ethanol evolution and seed moisture content, which influences membrane assembly and enzyme hydration, and (3) to determine the effect of surface sterilization on the amount of ethanol produced by imbibing seeds.

Corsoy 79 soybeans which were surface sterilized and nonsurface sterilized were artificially aged by either a high moisture, high temperature method or a constant 12% moisture, high temperature method. Seeds were imbibed for periods up to 34 hours at which time germination was completed. Headspace over imbibing seeds was sampled and the course of ethanol evolution was measured for the duration of water uptake to radicle protrusion. Ethanol production increased as viability and vigor declined, and peak ethanol production increased with imbibition time, or at increased moisture levels, indicating that hydration of enzymes alone does not explain the pattern of evolution. Increased delay in the attainment of membrane integrity with declined viability and vigor and the resulting imbalance between glycolysis and the

citric acid cycle may explain the patterns of evolution observed. Increased ethanol production in surface sterilized seeds may be due to declined utilization by microorganisms or by effects on cell membrane integrity.

LITERATURE REVIEW

Introduction

Seed vigor declines during aging before viability. This loss of vigor, or aging, is evidenced by delayed germination, delayed emergence in the field, slower growth, and increased susceptibility to environmental stress. Little is known about the actual mechanism of aging, but it has been shown that the metabolic production of ethanol increases with a reduction in seed vigor. Ethanol production has been shown to result from conditions which impose a stress on cell membranes including anoxia, aging treatments, low temperature, lipid peroxidation, and the use of metabolic inhibitors. The lipids and proteins of membranes become unstable under these conditions and the consequence is a loss of integration between anaerobic and aerobic respiration which leads to the production of ethanol. The concentration of ethanol produced is not the primary cause of loss of vigor, but is indicative of the occurrence and extent of anaerobic respiration. Ethanol measurements in imbibing seeds may provide a valuable tool in predicting germination and vigor.

Germination

Seed germination begins with the uptake of water. Water uptake, or imbibition, is accompanied by the activation of enzymes, the start of active metabolism, and the leakage of solutes (Simon and Mills, 1983). The rate of imbibition depends on the composition of the seed, the permeability of the seed coat to water, and the availability of water in the environment (Mayer and Poljakoff-Mayber, 1982). Cseresnyes and Vorenvinci (1984) showed that the amount of water taken up by soybean seeds was affected by the amount of available water in the imbibition substrate and the temperature of imbibition. A triphasic pattern of water uptake has been outlined for seeds placed in distilled water under optimum germination conditions (Beweley and Black, 1983). Initial imbibition, Phase I, is a physical process related to the properties of seed colloids and matric potential and not to the viability of the seed (Waggoner and Parlange, 1976). In soybeans, seed imbibition is characterized by a simultaneous entry of water and a swelling of seed polymers with cell walls and proteins the major components contributing to swelling (Leopold, 1983). Air-dry seeds have a very low water potential and so imbibe water readily since the water potential gradient between the seed and imbibing medium is usually quite high. Rapid initial imbibition rates slow as potential nears zero. Phase II is a lag period in water

uptake. The matric potential and osmotic potential are high. The extent of the lag period is dependent on the species of seed, individual seed, and temperature (Hegarty, 1978) and may not be present at all. As wetting proceeds, different parts of the seeds may progress through the phases at different rates, so considerable overlapping may occur (Berrie, 1984). Dead and dormant seeds may attain hydration to the level of Phase II, but only germinating seeds progress to Phase III which is associated with radical protrusion and further water uptake. Water uptake in Phases I and II is due to diffusion, while further uptake is energy dependant and retarded by metabolic inhibitors (Morohashi and Shimokoriyama, 1972b). During the final phase of hydration, there is an increase in the water content of wetted areas and osmotic potential is satisfied (Harper and Benton, 1966). A seed is considered by physiologists to have germinated when the radicle breaks through the outer seed coverings (Berrie, 1984).

When seeds are moistened, there is a release of gas adsorbed within the seed which is a physical phenomenon occurring in both viable and nonviable seeds (Haber and Brassington, 1959). During the initial rapid, nonlinear imbibition there is also a rapid, nonlinear leakage of solutes from the seed (Leopold, 1980). Solutes leaked from seeds include electrolytes, sugars, amino acids, organic

acids, phenolics, phosphates and proteins (Simon and Mills, 1983 and Duke and Kakefuda, 1981). The amount of leakage increases when dry seeds and embryos imbibe, when temperatures are low, and seeds are of low vigor or mechanically damaged. Leakage is usually suppressed by the time embryos have reached a water content of 15 to 35% (Simon and Weibe, 1975 and Bramlage et al., 1978). There have been several hypotheses concerning the source of leached solutes (Simon and Mills, 1983). One of these, the presence of surface deposits on seeds dissolving away, can be discounted because leakage continues even after repeated rinsing. Some researchers feel that the rapid influx of water into a dry seed causes a physical disruption of cells which is responsible for the leakage observed (Powell and Matthews, 1981 and Tully et al., 1981). However, even when imbibition is slowed, minimizing damage from rapid water uptake, leakage continues at a slower rate, but over a longer time period, suggesting that leakage occurs in not just the superficial cells of the seed, but in all the cells (Simon and Mills, 1983). Simon and Harun (1972) found that if seeds were dried after the start of imbibition and then rewet, they again would lose solutes. From these observations, Simon (1974) has proposed a model for phase change in hydrating membranes to explain the leakage phenomenon. According to Simon (1974), membrane phospholipids in dry seeds are in a

hexagonal configuration. When seeds imbibe water, the membranes become hydrated and adopt a lamellar configuration at approximately 20-30% of seed fresh weight. Thermal analyses showed that a water content of 20% was necessary to create a hydrophilic environment around the membrane and stabilize the membrane lipids in a bilayer. At a water content of less than 20%, there is a change in phospholipid orientation so that the polar head groups arranged along water filled channels forming a hydrophobic matrix. The lipids and proteins associated with membranes assume configurations to minimize hydrophobic and maximize hydrophilic interactions (Chapman et al., 1967). In the time taken to reach the lamellar configuration there is a leakage of cell contents. This hypothesis explains many of the phenomenon observed during water uptake, but is open to some criticism. Simon based his model on work done with brain phospholipids (Luzatti and Husson, 1962) and in working with seed preparations Seewaldt et al. (1981) found that membranes from soybeans maintained a bilayer configuration regardless of the state of hydration. They saw differing dimensions of the bilayer, depending on the degree of hydration and the availability of free water in the seed. It is likely that even if a hexagonal configuration does not exist, that membrane structure is disorganized in dry seeds (Simon and Mills, 1983). Using electron microscopy, Morrison-Baird et

al. (1979) found that the plasma membrane changed extensively during the first twenty minutes of imbibition while much smaller changes were observed in other organelles. Chabot and Leopold (1982) showed a graded series in state of organization for soybean plasma membrane as a function of moisture. They found a clustering of vesicles and lipid bodies along the plasma membrane and felt that imbibition may involve incorporation of new proteins and lipids into expanding membranes. Nawa and Ashai (1973) found that the influx of water into the seed plays a role in the assembly of preexisting cytoplasmic proteins into the mitochondrial membranes in peas.

Currently, the most accepted model of membrane structure is the fluid mosaic model (Singer and Nicolson, 1972). The fluid mosaic model is that of a bilayer of amphipathic lipids (phospholipids, glycolipids, and sterols) imbedded with globular proteins which may be peripheral or integral. The lipids are composed of polar, hydrophilic heads and nonpolar hydrophobic tails of hydrocarbons arranged so that the heads are associated with the aqueous phase and the tails are within the bilayer. Both the lipids and the proteins are able to diffuse laterally. The major phospholipids found in the plasma membrane and mitochondria are phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol. The most common fatty acids in plant phospholipids

contain sixteen or eighteen carbons (Simon, 1974). There are three major functions of cell membrane lipids (Raison, 1980). These include the formation of a selectively permeable barrier to two predominantly aqueous compartments, to provide a stable fluid medium for proteins that catalyze reactions and to provide a flexible boundary between the cell and its surroundings. The bilayer undergoes a physical change from fluid flexible crystalline which is associated with normal membrane function to a solid gel structure when the temperature drops below a narrow range. This phenomenon is known as phase transition (Goodwin and Mercer, 1983). The physical state of membrane lipids affects the structure and function of membrane proteins. Phase transition in membranes has been associated with permeability changes in rewetting dry tissue, chilling injury, leakage from lipid depleted tissues, and lipid peroxidation (Simon, 1974). Membrane phase changes, as measured by electron spin resonance, occur in the membranes of the mitochondria, chloroplasts, glyoxosomes and protoplastids. Phase transition occurs over a temperature zone, not at an exact point and is reversible. The temperature at which phase transition occurs increases with an increase in fatty acid chain length and decreases with increased fatty acid saturation. Membrane surface area decreased when the membrane phase changed from liquid crystalline to gel (Christiansen, 1984). A change in phase

can be observed as a change in the kinetic properties of membrane bound enzymes (Raison et al. 1971). Raison and Chapman (1976) found an increase in the Arrhenius activation energies of mitochondrial enzymes below a critical temperature and, using electron spin resonance spectroscopy, showed a concurrent change in the molecular ordering of mitochondrial membrane lipids. Their conclusion was that of a structural-function relationship between the molecular ordering of membrane lipids and a physiological response to a change in temperature. Changes in membrane lipids may alter the conformation of the active sites of membrane bound enzymes (Raison et al., 1971). Raison (1973) reasoned that the critical temperature for growth might be above the temperature at which initiation of phase change takes place. He speculated that the difference between chilling sensitive and tolerant plants may be due to the effect of temperature on membrane lipids.

Christiansen (1984) suggests that temperature alteration of lipids may be important in the function of plant cells at low temperatures. The mitochondrial membrane lipids of plants tolerant of low temperatures were more highly unsaturated than those of chilling sensitive plants. Pomeroy and Andrews (1975) found that cold hardening in wheat induced an increase in linolenic acid. Chemical inhibition of linolenic acid synthesis with BASF-338 blocked an increase in

cold tolerance (St. John and Christiansen, 1976). Temperature has been found to exert some control on fatty acid desaturation (Mazliak, 1979). Priestley and Leopold (1980b), however, found no differences in the major lipid components of seed membranes of chilling sensitive and insensitive soybeans.

Lyons (1973) proposed the following scenario for low temperature injury to plant cells. Cellular membranes in chilling sensitive plants undergo phase transition from flexible liquid crystalline to solid gel at temperatures critical for injury. This causes cracks or channels to form, increasing permeability so that cells leak, upsetting ion balance. The energy of activation (Ea) of membrane bound enzymes increases and reaction rate decreases, leading to an imbalance with nonmembrane associated enzyme systems. Both glycolytic and mitochondrial respiration rates decrease with temperature, but at phase transition, an increase in the Ea of mitochondrial enzyme mediated reactions causes major imbalance between glycolysis and the citric acid cycle, leading to a buildup of pyruvate, acetaldehyde and ethanol and a depletion of ATP. Prolonged exposure to low temperatures leads to injury and cell death. Phase changes may be a critical factor in germination and seedling injury under chilling stress, explaining the increased permeability of membranes and the metabolic imbalances observed during

imbibitional chilling injury of seeds. Failure to germinate below or above a certain temperature range may be a manifestation of the inability to cellular membranes to function properly as semipermeable membranes (Leopold, 1983).

Initial imbibition at temperatures below 15 degrees C causes injury in some seeds which may result in lack of germination or depression of seedling growth. This imbibitional chilling injury is apparently a phenomenon associated with the very early stages of hydration. Vertucci and Leopold (1983) found that chilling injury occurred with the initial wetting process and that low temperatures may decrease the ability of tissues to withstand expansion during hydration. Low temperature interference with membrane expansion in soybeans may cause lesions associated with imbibitional chilling injury (Willing and Leopold, 1983). Lesions may cause a disruption in membrane reorganization and respiratory dysfunction. Leopold and Musgrave (1979) found that soybean seeds respond to chilling stress by a loss of respiration through the cytochrome oxidase pathway. Both membrane damage and loss of sensitivity to cyanide occur in the first minutes of imbibition, and may be due to a temperature mediated disruption of membrane reorganization.

The regulation of imbibition rates by temperature is altered by the moisture content of the tissue. Rowland and Gusta (1977) found that low seed moisture when accompanied by

low imbibition temperatures reduced germination and seedling dry weight in peas and faba beans. Vertucci and Leopold (1983) found the largest temperature response was elicited from dry soybeans and the least from seeds with moisture contents of 68%. Similarly, Ashworth and Obendorf (1980) found that soybean axes of 6% moisture were injured when imbibed at 5 degrees C while axes of 17% moisture were not. Hobbs and Obendorf (1972) found that a seed moisture content of 13 to 14% protects against imbibitional chilling injury but does not prevent leaching. Soybean seeds with moisture contents of 6% were sensitive to cold temperatures during imbibition but if the same seed was imbibed first at 25 degrees and then moved to 5 degrees there was no reduction in survival (Obendorf and Hobbs, 1970). These researchers found that adjusting seed moisture content to 16% before seeding reduced imbibitional chilling injury and reduced leakeage of organic materials. Bramlage et al. (1978) found that embryos with a moisture content of 35 to 50% did not succumb to imbibitional chilling injury when imbibed five minutes at 2 degrees C. They suggested that imbibitional chilling injury was due to low temperature interference in membrane reorganization which caused internal disruption and solute leakage. The initial rate of leakage in peas at 25 degrees C was found to be higher that at 5 degrees but persisted for a longer period of time at the lower temperature. If seeds

were imbibed at 5 degrees after a period of imbibition at 25 degrees, the rate of leakage was at the rate measured for 25 degrees but if the seeds were first imbibed at 5 degrees and then 25 degrees, leakage continued for 48 hours at the 5 degree rate (Marbach and Mayer, 1985). These results were interpreted as showing that membrane integrity was established at the 25 degrees C imbibition temperature, but was slowed at 5 degrees C. Tully et al. (1981) found that seeds that have a slower imbibition rate as in peas are not as sensitive to imbibitional chilling injury as seeds which take up water more rapidly such as soybeans. The use of osmotic solutions to slow imbibition rates can reduce the damage caused by rapid imbibition (Perry and Harrison, 1970). Pre-equilibration of soybean embryonic axes to a higher moisture content or use of polyethylene glycol can reduce leakage by reducing the rate of water uptake and allowing more time for membrane function to become established (Woodstock and Taylorson, 1981b).

During the initial stages of water uptake there is a resumption of growth in the quiescent seed. During rapid initial imbibition, membrane reorganization takes place, enzymes become activated, and there is a rapid increase in respiratory activity (Parrish and Leopold, 1977). Early metabolism is a function of seed water content. Opik and Simon (1963) found that the rise in respiration during

imbibition was due to activation of pre-existing enzymes by hydration in Phaseolus cotyledons. Nawa and Ashai (1973) found that the activity of cytochrome oxidase and malate dehydrogenase depended on hydration. Pea seeds imbibed at 2 degrees C for 24 hours had the same enzyme activity as those imbibed 5 to 6 hours at 25 degrees. Edwards (1976) found that fixation of labelled carbon dioxide and utilization of labelled acetate increased exponentially with seed moisture in charlock. At seed moistures of less than 8%, Vertucci and Leopold (1984) found no measureable respiration in soybeans due to lack of enzyme activity. At moisture contents between 8 and 24%, oxygen uptake was still somewhat restricted, but at moisture contents of greater than 24% there was a rapid rise in oxygen uptake. Increased resistance to imbibitional damage with increased seed moisture content indicated that membrane integrity was becoming established.

Opik and Simon (1963), Kolloffel (1967), Morohashi and Shimokoriyama (1972a) and Ching (1973b) have found that respiration in imbibing seeds appears to be triphasic. The duration of each phase differs with species and experimental conditions. During phase I there is a rapid rise in oxygen uptake and pre-existing enzyme systems are activated. There is an increase in ATP content for synthesis and provision of substrate for respiration and protein synthesis. Phase II is termed the lag phase and oxygen uptake remains constant or

may even decrease slightly. Water content does not change substantially and Ching (1973b) found that during this phase, synthesis of enzymes and organelles for the catabolism of storeage reserves occurred. Phase III begins another period of increasing oxygen uptake and also a slow increase in water content which is metabolic in nature and can be stopped by metabolic inhibitors or low temperatures (Morohashi and Shimokoriyama, 1972a). Protein synthesis occurs and the phase is terminated by radicle emergence. Initial radicle expansion does not require cell division but further growth involves both cell elongation and cell division (Beweley and Black, 1983).

The increase in respiration with increasing moisture content that is observed during germination has been attributed to increases in the number of mitochondria, the mitochondrial protein content, enzyme activity, cytochrome content, and mitochondrial DNA (Solomos et al., 1972). Sato and Ashai (1975) found that immature mitochondria in dry pea seeds became active and stable through assembly of protein into their membranes. The mitochondria in dry seeds had few cristae and those of imbibed seeds had many cristae and increased biological function. Nawa and Ahshai (1971) found that mitochondrial protein and phospholipid content increased in pea cotyledons concurrent with an increase in respiratory activity and concluded that maturation of existing

mitochondria was responsible. Morohashi (1986b) found that the mechanism involved in the rise in respiratory activity may be dependant on the type of storeage reserve in the seed. In lipid storing seeds he found an increase in the activity of mitochondria and glyoxosomes due to de novo protein synthesis while in starch storing seeds there was activation of pre-existing mitochondria.

The main respiratory pathways in germinating seeds are glycolysis, the tricarboxylic cycle, the electron transport chain and the pentose phosphate pathway (Mayer and Poljakoff-Mayber, 1982). Glycolysis and the pentose phosphate pathway are the major sources of pyruvate in plants which may enter the tricarboxylic cycle or fermentative pathways. In addition, glycolysis is the source of substrate level phosphorylation and NADH production. The pentose phosphate pathway provides NADPH for fatty acid synthesis and membrane synthesis. The citric acid cycle is linked to the electron transport chain and provides energy and carbon skeletons for synthesis.

Kolloffel and Sluys (1970) felt that the biochemical integrity of the electron transpost apparatus determined respiratory capacity in peas during early germination. Morohashi and Shimokoriyama (1974) also felt that the citric acid cycle and electron transport chain were rate limiting in early germination of Phaseolus mungo because glycolytic

inhibitors such as NaF and IAA had no effect on respiration rates.

Despite work by Yentur and Leopold in 1976 showing that up to 50% of the initial respiration in germinating soybeans was cyanide resistant, more recent work has shown that the capacity for alternate respiration varies widely within and among species, and is not necessary for germination (Morohashi, 1986a). Dizengremel and Tuquet (1984) found that oxidative phosphorylation and the cyanide sensitive pathway were functional from the start of imbibition and that the capacity for the alternate pathway developed only as oxygen uptake increased. Early respiration in chick peas (De la Fuente-Burguillo and Nicolas, 1974), soybeans, and mung beans (Siedow and Girvin, 1980) has been shown to be cyanide sensitive.

Vigor

Seed vigor is an important aspect of seed quality. There is considerable disagreement over how to define the term vigor and even more controversy on how it should be measured. Abdul-Baki (1980) points out that vigor can be considered on an individual seed level in which one seed produces one normal seedling or on a basis of a population of seeds, producing rapid, uniform germination of a large number of individuals. The International Seed Testing Organization defines vigor academically: "Seed vigor is the sum total of

those properties of the seed which determines the potential level of activity and performance of the seed of seed lot during germination and seedling emergence". The other major seed testing organization, the Association of Official Seed Analysts, defines vigor on an operational level: "Seed vigor comprises those seed properties which determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of field conditions."

Following physiological maturity (the attainment of maximum dry weight), there is an irreversible decline in vigor and viability. Loss of viability is normally preceded by a loss of vigor. Individual seeds lose vigor at different rates (Anderson and Baker, 1983). The exact mechanism of deterioration is unknown, but seed organelles change due to changes in the internal cell enviroment (Anderson, 1973).

During quiescense, loss of vigor occurs at a rate determined by genetic makeup and the interior and exterior seed environment (Abdul-Baki and Baker, 1973). Villiers and Edgcumbe (1975) found that dry seed had a high mutation rate and accumulated nuclear damage in storage while seed stored in an imbibed state sustained few chromosomal aberrations. They hypothesized that the imbibed seed maintained macromolecules and were able to repair damage and so, accumulated little structural damage. Pulse-chase experiments in imbibed, dormant embryos of Avena fatua showed

a continual turnover of membranes. Degenerative changes were resisted by this maintenance of cellular integrity. An impairment of turnover in dry seeds may lead to a loss of vigor and viability (Cuming and Osborne, 1978).

The loss of germination is the final consequence of deterioration and is preceded by biochemical and physiological changes. Delouche and Baskin (1973) outline the progression of changes that precede seed mortality. The first step in deterioration is the degradation of cellular membranes and loss of permeability, followed by impairment of energy yielding and biosynthetic mechanisms, reduced respiration and biosynthesis, slower germination and seedling growth, reduced storage potential, slower growth and development of autotropy, less uniformity in growth and development in the seedling population, increased susceptibility to environmental stress, reduced stand and production potential, increase in abnormal seedlings, and finally, loss of viability.

Manifestations of vigor loss may be physiological or biochemical. Biochemical changes associated with deterioration may be genetically controlled such that a chemical reaction in one part of the cell affects reactions in other parts so that a change or malfunction could lead to a slowing or halt in growth. Biochemical changes include compositional changes in lipids, fatty acids, and proteins,

increased permeability of cellular membranes, and a decline in respiratory metabolism (Abdul-Baki and Baker, 1973). Physiological changes include rate and uniformity of seed germination and seedling growth, rate and uniformity of seedling emergence and growth in the field, and the emergence ability of seedlings under unfavorable conditions (Perry, 1978). Seeds with reduced vigor are more susceptible to colonization by microorganisms (Harmon and Granett, 1972 and Cherry, 1983).

Constitutive changes during aging include an increase in free fatty acids and inorganic phosphate levels, and a decrease in phospholipid, soluble sugars, and protein levels (Anderson, 1973). Francis and Coolbear (1984) found that deterioration caused a progressive decline in the phospholipid content of tomato seeds with the loss being primarily due to the breakdown of phosphatidyl choline. The major location of plant phospholipids is in membranes. Koostra and Harrington (1969) found that the amount of phosphorus containing lipids was reduced in artificially aged seeds and that oxidative products of four phospholipids, primarily phophatidyl choline, increased. Aged seeds have a low rate of *in vivo* protein synthesis. Anderson and Baker (1983) found quantitative but not qualitative changes in proteins synthesized, while Chauhan et al. (1985) found that the electorphoretic protiles of proteins from aged seeds that

had been germinated for 24 and 48 hours contained fewer bands than did unaged seeds.

As vigor is lost, many of the biochemical changes that occur have been attributed to membrane changes. Increased permeability, reduced phosphorylative capacity of mitochondria, reduced synthesis and a decrease in phospholipids have all been shown to accompany vigor decline in seeds (Abdul-Baki and Baker, 1973). Parrish et al. (1982) found that the ability of the cell to develop turgor was directly related to membrane integrity. A decline in turgor was measured in aged seeds prior to a decline in germinability and growth. A decline in the semipermeable nature of the plasma lemma and solute leakage can lead to a loss of turgor. Perry and Harrison (1970) found increased electrolyte loss from low vigor seeds as compared to high vigor seeds. Leopold (1980) found a tenfold increase in solute leakage in dead seeds when compared to viable seeds. The quantity of solutes leaked has been negatively correlated with seed viability and vigor (Simon, 1974, Simon and Harun, 1972, Parrish and Leopold, 1978, and Yaklich and Abdul-Baki, 1975). Matthews and Bradnock (1968) found a significant negative correlation between field emergence and the amount of electrolyte leakage from imbibing pea seeds. The mortality of low vigor seeds in cool spring temperatures after heavy rains has been partially attributed to exudates

from seeds which may attract soil borne pathogens and cause emergence failures (Perry and Harrison 1970). Cuddy (1976) found that the leakage of sugars increased the activity of soil fungi and a relationship between leakage of carbohydrates and the incidence of disease in the field was found by Short and Lacy (1976).

The lipids and proteins of membranes are unstable under stress conditions including low moistures and temperature extremes (Abdul-Baki, 1980). Sensitivity to chilling is determined in part by seed quality (Pollock and Toole, 1966). Deterioration in soybeans increases susceptibilty to water uptake injury at all temperatures and altered subsequent metabolism (Woodstock and Tao, 1981).

The membranes of the mitochondria are of particular interest because it is within the matrix that enzymes of the citric acid cycle are located and oxidative phosphorylation takes place. Abdul-Baki (1980) states that "vigor may be viewed as a function of the time required by the mitochondria to become more efficient in oxidative phosphorylation and their membrane system to become more highly developed than that in dry seeds." Respiration provides energy for the germinating embryo so respiratory activity is closely associated with vigor and viability. The coordinated activity of many enzymes is necessary for efficient function, so respiratory measurements are a good assessment of overall

metabolic activity. Deterioration from many kinds of injury can be detected, including damage from freezing, heat, drying, mechanical damage and chilling injury (Woodstock, 1973). Processes involving respiration during imbibition influence subsequent development (Woodstock and Grabe, 1967). Glucose utilization declines before viability (Anderson and Baker, 1983). Low quality seeds mobilize, interconvert, and utilize stored reserves at a slower rate than do high quality seeds (Burris et al., 1969). Respiratory energy is necessary for protein synthesis, and incorporation of 14-C-leucine closely follows oxygen uptake rates (Abdul-Baki, 1969). A positive correlation has been found between vigor and the uptake and utilization of 14-C labelled leucine and glucose (Abdul-Baki and Baker, 1973). Deterioration in seeds has been shown to result in a decline in oxygen uptake, increases in respiratory quotients, a decrease in phosphorylative effiency and respiratory enzyme activity, changes in ATP levels, an increase in the cyanide resistant respiratory pathway, and production of ethanol (Woodstock et al., 1984).

Decline in oxygen uptake has been shown to accompany loss of vigor from different causes. Woodstock et al. (1985) demonstrated a decrease in oxygen uptake and increased respiratory quotients in weathered lots of cottonseed. Respiratory rates, as approximated by oxygen uptake during the first 24 hours of germination, were shown to be

positively correlated with final germination, seedling growth and field emergence in corn (Woodstock and Grabe, 1967). Woodstock and Feeley (1965) found that respiratory rates after two hours of germination could detect damage from heat, freezing, or irradiation. Woodstock et al. (1984) showed that the mitochondria from low vigor soybean axes had lower oxygen uptake rates, lower ADP:O ratios, and lower respiratory control ratios than high vigor axes.

Respiratory quotients (RQ= volume of carbon dioxide produced/ volume of oxygen taken up) tend to increase in deteriorated seed. This can be due to carbon dioxide being produced faster than oxygen is taken up, or an increase in carbon dioxide production accompanied by a declined or unchanged oxygen uptake (Anderson and Baker, 1983). Woodstock et al. (1984) attributed an increase in the RQ of deteriorated soybeans to a relatively greater decrease in oxygen uptake than an increase in carbon dioxide production. Pesis and Ng (1984a) found an increased RQ due to higher carbon dioxide production associated with ethanol production in low vigor muskmelon seeds. They pointed out that the pentose phosphate pathway and glycolysis both produce carbon dioxide, but do not consume oxygen. They found that oxygen uptake is less than carbon dioxide evolved. Abdul-Baki (1980) states that RQ can be used as a measure of mitochondrial recovery in early imbibition; the seed lots

with the lowest RQ in early imbibition are the most vigorous seed lots. Woodstock and Grabe (1967) found a high negative correlation between increased RQ and seedling growth.

Woodstock (1973) suggests that injurious seed treatments are more serious for mitochondrial than glycolytic pathways since the mitochondria require a high level of subcellular integration between membrane structure and enzyme function. Woodstock and Taylorson (1981a) proposed that as a result of deterioration there is an imbalance in the activity of glycolysis relative to the citric acid cycle. The greater activity of glycolysis results in a higher level of carbon dioxide produced compared to oxygen uptake, resulting in an increased RQ. During early imbibition glycolytic enzymes are activated earlier than those of the mitochondria (Morohashi and Shimokoriyama, 1975b), resulting in a higher initial RQ. Collins and Wilson (1972) have shown that the citric acid cycle was not active before 30 minutes of imbibition. The response to the resulting buildup in pyruvate is an increased RQ and production of ethanol (Beevers, 1961).

Changes in ATP levels have been associated with vigor decline in many different seeds. Ching (1973a) found a high correlation between ATP content, seed weight, and seedling size in crimson clover, rape, and ryegrass (protein, fat, and starch storing seeds, respectively). She found that ATP content in imbibing seeds was reduced as a result of aging

stress. Yaklich et al. (1979) found that the capacity of soybean axes to convert adenine and adenosine to ATP was lower for deteriorated seeds than high vigor seed. These researchers found no correlation between ATP content and field emergence. Anderson (1977) suggested that the ability to accumulate adenylates during early germination was related to vigor. More recently, however, Perl (1986) concluded that ATP accumulation was not a good measure of seed quality, because accumulation may result from a high synthetic rate (high vigor) or a low utilization rate (low vigor). Perl presents evidence for ATP production from a synthetic cycle different from oxidative phosphorylation in which malate is used as a carbon source and pyruvate is an endproduct. NADH may stimulate ATP production from AMP since at the beginning of germination AMP levels were found to decrease and ATP levels to increase.

The increase of the cyanide resistant or alternate pathway in deteriorated seeds has been reported by Priestley and Leopold (1980b). Unaged seeds utilize primarily the cyanide sensitive pathway for electron transport, especially the embryonic axes. Alternate pathway transport in cotyledonary tissue is utilized, possibly to provide carbon skeletons for seedling growth. After accelerated aging, there is an increased insensitivity to cyanide, indicating that more electron transport is occurring via the alternate

pathway (Leopold and Musgrave, 1980). This means that little or no phosphorylation is occurring and there is a depletion of ATP.

Causes of vigor loss or the lack of vigor may be genetic, physiological, pathological, or mechanical (Heydecker, 1969). The maximum germinative capacity of a seed is genetically determined, but whether that capacity is expressed depends on a number of factors. Premature harvest, post-harvest weathering, and conditions of storage can all affect seed vigor. Environmental variables during seed development can affect subsequent seed quality. According to Delouche (1980) soil fertility, moisture content, and temperature during development can affect seed quality. Other factors include aeration levels, pathogen content and activity, and structural properties which affect variables such as crusting liability and seedling penetration (Hegarty, 1978). Changes in seed quality are primarily due to four factors: time, temperature, relative humidity, and oxygen level (Roos, 1980). A decrease in seed moisture content of 1% can increase the life span of seed in storage by a factor of two (Harrington, 1973). Storability is an aspect of seed quality that reflects prestorage treatment and the history of the seed since lots of the same kind, variety, age, and viability do not store equally well (Delouche and Baskin, 1973). A close relationship exists between the response after

accelerated aging and storage life.

Accelerated aging was first developed to estimate storage potential of seeds. It has since been used to predict seedling stands and estimate vigor in a wide range of crop seeds, and is a valuable research tool used to obtain seeds of different vigor levels. The test exposes seeds to high moisture (greater than 90% relative humidity) and high temperature (40-45 degrees C) for short periods of time which speeds deterioration of seed quality. Delouche and Baskin (1973) proposed that the decrease in viability following accelerated aging is proportional to the initial physiological potential of the seed. The advantages of accelerated aging include a decrease in the time taken to obtain sampes as well as the elimination of physiological variables involved in collecting seeds from different seasons. A greater degree of genetic uniformity is also obtained (Berjak and Villiers, 1972c). Increasing evidence, however, has shown that the mechanism of deterioration involved in accelerated aging may not be the same as that involved in natural aging. Berjak and Villiers (1972b) found an unusual pattern of senescence following artificial aging in corn. They observed an acceleration of genetically controlled aging processes and that degenerative changes were not limited to the most mature cells. Abdul-Baki and Anderson (1970) found that the leaching of sugars in seeds

increased with seed age and with mechanical damage, but remained unchanged by accelerated aging unless there was a severe reduction in viability. They suggested that the physiological changes incurred during accelerated aging were not the same as those which occurred as seeds aged naturally. Priestley and Leopold (1979) found that soybean seeds subjected to accelerated aging lost vigor and viability without a change in polyunsaturatation of fatty acids whereas seed in long term dry storage incurred autoxidation of polyunsaturated fatty acids. Koostra and Harrington (1969) felt that artificial aging amplified small differences in phospholipid changes incurred during natural aging. Anderson and Baker (1983) suggest that the decreases in saturated fatty acids found in some aging research may be associated with high temperature rather than deterioration. The conditions of accelerated aging are also highly conducive to the growth of microorganisms which add a variable not necessarily present in natural aging (Anderson and Baker, 1983).

The exact mechanism for the vigor decline that precedes loss of viability is unknown. A mechanism for aging should explain the deleterious effects of increased moisture, temperature and oxygen, the extended life span of imbibed seeds, the appearance of chromosomal aberrations, and the reduction in vigor and delay in germination noted in aged

seeds (Villiers, 1973).

One theory for the deterioration of seeds is the autoxidation of lipids. Lipid peroxidation mediated by free radicals may contribute to the aging process by damaging the cellular membranes of the mitochondria, endoplasmic reticulum, and lysosomes. Autoxidized linolenic and linoleic acid have been found to have mutagenic activity (Yamaguchi and Yamashita, 1979). Koostra and Harrington (1969) suggest that this series of events leads to deterioration in cells. Unsaturated fats react in the presence of metal ions to form free radicals. Free radicals reacting with oxygen form hydroperoxides which in turn form carbonyls. Carbonyls reacting with proteins can cause inactivation of enzymes, membrane injury, and denaturation of histones. The subcellular membrane model of an unsaturated lipid bilayer with associated globular proteins would be susceptible to attack by free radicals and membrane damage could be incurred at the time of imbibition (Berjak, 1978). Senaratna et al. (1985) found an increase in the free fatty acid:phospholipid ratio in isolated membranes exposed to free radicals, suggesting that free radical induced deesterification of membrane phospholipids. During imbibition, when degraded membranes of vacuoles or lysosomes become hydrated, they may release hydrolytic enzymes including ATPases, RNAases, and lipases which cause further damage (Villiers, 1973).

Reactions with nucleic acids could conceivably cause chromosomal aberrations. The production of free radicals and hydroperoxides is autocatalytic because each cleavage of a double bond produces two free radicals (Harrington, 1973). Lipid autoxidation is increased by high temperatures and inhibited by removing oxygen. It may be that this process is only important at low seed moistures of four to six per cent. At higher moistures, water may act as a buffer between macromolecules and reactive compounds. Phospholipids and tocopherols are antioxidants. In dry seeds there are no active enzyme reactions to produce new tocopherols as they are used up. Villiers (1973) found that imbibed seeds retained their germinative capacity longer than dry controls and postulated that the imbibed seeds were able to initiate repair and replacememt of damaged organelles.

If the postulated autoxidative reactions occur, highly unsaturated fats should disappear as seeds age (Harrington, 1973). In pea seeds, the primary fatty acids Harrington found were palmitic, stearic, oleic (all saturated) and linoleic and linolenic (unsaturated). After artificial aging treatments, he found a rapid decrease in linolenic acid which paralleled loss in vigor in pea axes. There were also declines in the less reactive linoleic acid levels. St. Angelo and Orly (1983) demonstrated lipoxygenase formation of polyunsaturated fatty acid hydroperoxides by adding oxygen at

double bonds. Lipoxygenase is specific; it acts on cis,cis 1,4-pentadiene structures such as linoleic, linolenic, and arachadonic acids. Harmon and Mattick (1976) found a positive correlation between the disappearance of linolenic acid and the loss of vigor and seed death in peas and concluded that the formation of free radicals and oxidation of unsaturated lipids could be responsible. In contrast to these findings, are those of Priestley and Leopold (1979). They found that there were minimal changes in the degree of unsaturation of fatty acids in artificially aged soybean seed. When they compared seed lipids in soybeans that had been artificially aged with lipids from naturally aged soybeans, they found a decrease in the proportion of polyunsaturated fatty acids in naturally aged seed, but not in the seed that had been artificially aged even though the seeds were of comparable vigor and viability (Priestley and Leopold, 1983).

Loss of vigor prior to viability may be due to impaired DNA templates and restricted RNA transcription which can result from chromosomal aberrations and point mutations (Roos, 1980). Osborne et al. (1974) found that as per cent viability declined, ribosomes were impaired and DNA degraded. A decrease in transferase activity and an increase in DNAse and RNAse activity were found in low vigor seeds. Loss of viability in storage has been associated with an increase in

chromosomal aberrations (Abdalla and Roberts, 1968 and Dourado and Roberts 1984). Abdalla and Roberts (1968) found that increases in temperature, seed moisture content, or oxygen level could increase the rate of loss of seed viability in storage and lead to the accumulation of aberrant embryonic cells. They hypothesized that cell death is the result of accumulated nuclear damage and that the frequency of occurrence of aberrant cells can be used as an index of nuclear damage. A high correlation was found between seed viability and chromosomal damage. Nonlethal damage to DNA can lead to the formation of defective RNA and, ultimately, defective proteins (Berjak and Villiers, 1972c). Slower germination rates and reduced protein synthesis rates are characteristic of aged embryos. Osborne et al. (1974) found no in vivo protein synthesis, an absence of respiratory activity and abnormal membrane ultrastructure in aged nonviable rye embryos. Abdul-Baki and Chandra (1977) found that although rapid aging did not affect total nucleic acid content of embryonic axes, de novo nucleic acid synthesis decreased which was correlated with declined protein synthesis, germinability, and growth.

Heydecker (1969) suggests that chromosomal aberrations and mutations may play a minor role in seed death and that low moisture storage may lead to an accumulation of toxic substances (possibly abscissic acid) and a breakdown of

triggering mechanisms for germination (giberellins, ethylene, and kinetin). Although it has been suggested that automutagenic substances may accumulate during storage, Abdalla and Roberts (1968) failed to find any mutagens in aged seed lots of peas, beans or barley. An increase in DNAse activity due to losses of inhibitors has also been suggested as a mechanism for aging related genomic changes (Roberts, 1973). Ching and Schoolcraft (1968) attributed increases in permeability, free amino acids and inorganic phosphate in aged seeds to increased activity of proteases, phytase, and phosphatases.

Harrington (1973) postulated that protein denaturation could be one of the causes of aging. Denaturation of chromosomal histones could cause DNA activity to be blocked, enzyme activity could be affected, and membrane permeability could be increased by denaturation of constituent proteins. Roberts (1973) hypothesized that changes in enzyme activity may be due to denaturation or decreased synthetic activity.

Berjak and Villiers (1972c) concluded that loss of viability was due to damage to nucleic acids, membrane components, and enzymes. They placed aged seeds into three groups based on the extent of damage incurred. The first group sustained damage to the membranes of cytoplasmic organelles but the damage was repaired at imbibition. Delayed germination resulted while mitochondrial and plastid

membranes were repaired. During this recovery period, there was a temporary increase in DNA replication, production of RNA and proteins, and a proliferation of mitochondria and endoplasmic reticulum (Berjak and Villiers, 1972a). The second group of seeds also sustained membrane damage, however, there was no repair and a failure of control at the molecular level resulted. In the third group of seeds there was total degeneration of the seeds following imbibition. Berjak and Villiers (1972c) found that embryos which had lost germinability were still able to continue some metabolic processes. They found endoplasmic reticulum synthesis, polysome formation, and an ability to incorporate labelled uridine and leucine, but no cell division was observed and there was no radicle protrusion. Harrington (1973) has also reported enzyme activity in nongerminable seeds and McLeod (1952) found a lag between loss of germinative capacity and the loss of ability to reduce tetrazolium chloride salts in barley. Amylose, proteinase, maltase and phosphatase activity continued long after germinative capacity was lost.

Ethanol Production

Ethanol production in seeds and seedlings is well documented. Anaerobic respiration results in an accumulation of fermentation products, low ATP yields, and depletion of organic compounds (Kawase, 1981). Ethanol is one of the primary products of fermentation.

Glycolysis and fermentation are identical processes through the production of pyruvate. In the so-called priming reactions, hexose sugars are converted to triose phosphate and in the energy conserving reactions, pyruvate is formed and ATP and NADH are generated (Hall et al., 1981). Aerobically, pyruvate enters the citric acid cycle while anaerobically it is converted to lactate or ethanol. An increase in glycolytic activity during anoxia results in decreased oxygen uptake and subsequent oxygen depletion, decarboxylation of pyruvate to acetaldehyde, and conversion of acetaldehyde to ethanol. Alternatively, pyruvate can be reduced to lactate. Glycolysis and glucose utilization are stimulated by anoxia. This phenomenon is known as the Pasteur effect. The acceleration of glycolysis in anaerobic conditions is associated with a decrease in ATP, 3-phosphoglycerate, and phosphoenolpyruvate and while levels of ADP, fructose 1,6-bisphosphate and inorganic phosphate increase, a drop in energy charge is present.

Phosphofructokinase (PFK), an allosteric enzyme which catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, is the committed step in glycolysis and is an important metabolic control site. PFK is inhibited by ATP and citrate and stimulated by ADP, AMP, and fructose 6-phosphate. Increased energy demand under anaerobic conditions results in reduced ATP levels. High levels of ATP lower the

affinity of PFK for fructose 6-phosphate. Since the inhibitory effect of ATP can be reversed by AMP, enzyme activity increases when the ATP/AMP ratio is low. Citrate, an intermediate in the citric acid cycle, also inhibits PFK, signalling that carbon skeletons for biosynthesis are not needed. Citrate enhances the inhibitory effect of ATP.

Recently, the presence of a pyrophosphate linked phosphofructokinase (PFP) which catalyzes the reversible reaction of fructose 6-phosphate to fructose 1, 6-bisphosphate has been demonstrated in plants. Fructose 2, 6-bisphosphate functions as a regulatory metabolite and is involved in the interconversion of two molecular forms of PFP. A large molecular form has a high ratio of glycolytic to gluconeogenic activity while a small form has the reverse effect. Fructose 2, 6-bisphosphate promotes conversion of the small form to the large form, stimulating flux through glycolysis (Huber, 1986).

Pyruvate kinase (PK) catalyzes the production of pyruvate from phosphoenolpyruvate. PK is inhibited by ATP and stimulated by ADP and citrate. Although PK catalyzes an irreversible reaction, allsteric kinetics for the plant enzyme have not been demonstrated, and PK probably acts synergistically with PFK in regulating glycolysis (Goodwin and Mercer, 1983).

Pyruvate decarboxylase catalyzes the decarboxylation of

pyruvate to acetaldehyde. This enzyme requires thyamine pyrophosphate and Mg^{++} as cofactors and the reaction is exergonic, making this step largely irreversible. Acetaldehyde is reduced to ethanol by alcohol dehydrogenase (ADH), an enzyme containing zinc at its active site. The reaction involves a nicotinamide cofactor (NADH) and so, is pH dependent. The anaerobic fate of pyruvate may therefore be determined by pH, pyruvate concentration, and ATP concentration (Davies et al., 1974). Davies (1973) proposed that activation of PFK during anoxia is due, in part, to a lowering of cytoplasmic pH. The pH optima of pyruvate decarboxylase (PDC) and lactate dehydrogenase (LDH) are 6.0 and 7.0, respectively. Increased pyruvate levels stimulate ethanol production even when oxygen is not limiting (Beevers, 1961). Hence, the low energy yield of fermentation which results in declined ATP levels may activate LDH. Increased lactate levels could cause the pH to fall, stimulating PDC activity. These hypotheses are substantiated by Crawford (1977) who found that under anoxic conditions maximum ethanol levels occur hours later than maximum lactate levels and by Boyle and Young (1983) who found that high LDH activity preceded PDC and ADH activity.

During anoxia, the enzyme activity of PDC, ADH, and LDH increases. These increases may be partially due to the activation of pre-existing enzymes since both ADH

(Kolloffel, 1968) and LDH (Leblova et al., 1974) have been extracted from unimbibed seeds. Both of these enzymes have been found to be induced by substrate buildup indicating a coarse control. Leblova et al. (1974) found that both substrate addition and induction of anaerobiosis increased ADH activity and App and Meiss (1958) found that the application of ethanol induced ADH activity in rice. McManmon and Crawford (1971) found that acetaldehyde induced ADH in flooding sensitive plants. John and Greenway (1976) found that cyclohexamide inhibited both ADH and pyruvate decarboxylase but did not interfere with de novo synthesis of these enzymes. Pesis and Ng (1984b) found that ADH exists in seeds in amounts greater than needed for anaerobic respiration and concluded that no de novo synthesis occurred.

Research indicates that control of ethanol production may be via pyruvate decarboxylase. John and Greenway (1976) found high levels of ADH and pyruvate decarboxylase in rice several days after aerobic conditions were restored even though no more ethanol was produced. Because pyruvate decarboxylase activity approximated observed ethanol production rates more closely than ADH activity, these researchers concluded that pyruvate decarboxylase controlled ethanol production since any acetaldehyde produced would have been converted to ethanol. Chang et al. (1982) found a positive correlation between ethanol production and pyruvate

decarboxylase activity and showed pyruvate decarboxylase to be the rate limiting step in ethanol production by sweet potatoes. They also found the enzyme to be stimulated by high levels of pyruvate and low pH resulting from organic acid production and increased carbon dioxide levels during anaerobiosis. Increasing evidence points toward fine as well as coarse control of pyruvate decarboxylase. The enzyme acts at a branch point in pyruvate metabolism which is often a point of fine control (Pradet and Bomsel, 1978). Wignarajah and Greenway (1976) demonstrated a sigmoidal saturation curve with positive cooperativity for pyruvate decarboxylase, and Chang et al. (1982) found NADH to be a stimulator of PDC activity. A high NADH/NAD ratio occurs during anoxia. John and Greenway (1976) postulated that fine control effectors were pyridine nucleotides and thiamine pyrophosphate (TPP). They also hypothesized that NADH could replace TPP anaerobically.

The accumulation of ethanol in plant tissues has phytotoxic effects. Alcohols act directly on the membrane lipid bilayers. Aliphatic alcohols produce changes in the structural arrangement of lipids by acting as lipophillic agents and affecting membrane fluidity. The maximum change elicited declines with increasing chain length and increases with concentration. In Tetrahymena cells grown in 1.6% ethanol, Nandini-Kishore et al. (1979) found a reduction in

16:1 and 16:2 fatty acids and an increase in 18:2 fatty acids. The incorporation of more fluid unsaturated fatty acids into membrane lipids was shown by freeze fracture electron microscopy. Similar results were found using E coli (Ingram, 1976). Membrane proteins are also affected by ethanol. ATPase in the plasma membrane has been shown to be inhibited by ethanol (Grisham and Barnett, 1973) and Herscovits and Jaillet (1969) showed denaturation of myoglobin by alcohol. There is a close relationship between membranes and several enzyme systems, implying that the effect of ethanol on the physical state of membranes could directly affect enzyme function (Raison, 1973). Anaerobically grown rice seedling coleoptiles exhibited a reduced capacity for oxygen uptake and depressed cytochrome oxidase activity upon reestablishment of aerobic conditions (Opik, 1973).

Electrolyte leakage from seeds usually indicates membrane damage. Soybean seeds treated with alcohol expressed increased solute leakage which was related to a decline in germination and a decrease in axis growth (Priestley and Leopold, 1980a). Chang et al. (1983) found that sweet potato tubers sustained membrane damage after anoxic exposure as evidenced by increased electrolyte leakage. They found that ethanol alone had no direct effect on membrane permeability annd postulated that permeability may be indirectly affected

by the influence of ethanol on synthesis and repair of membrane components. Molecular oxygen is necessary for the synthesis of unsaturated fatty acids. Hethrington et al. (1982) found no de novo synthesis of unsaturated fatty acids in Iris. De Villiers et al. (1980) found that 3% ethanol inhibited respiration and synthesis of RNA, proteins and lipids in Phaseolus vulgaris hypocotyl cells. Another apparently membrane related anomaly is the prevention of the action of the far red absorbing form of phytochrome in Rumex (Taylorson, 1984).

Endogenously produced ethanol is much more toxic than that applied exogenously (Crawford and Zochowski, 1984 and Nagodawithana and Steinkraus, 1976), and plants that are able to eliminate ethanol from their tissues are less likely to suffer from ethanol induced damage. Chang et al. (1982) found that the extent of flooding or soaking damage is dependant on the rate of ethanol production, the length of the lag period before accumulated ethanol is metabolized, and the extent that the produced ethanol is metabolized. Kolloffel (1968) found the disappearance of ethanol in germinating pea seeds to be accompanied by an increase in oxygen uptake and a fall in RQ to near unity. Cossins (1978) proposed that, initially, ethanol is converted back to acetaldehyde, then to acetone or acetate, and then to acetyl CoA which enters the citric acid cycle. Cameron and Cossins

(1967) found minimal amounts of labelled carbon dioxide in pea cotyledons incubated with 14^C labelled ethanol but considerable amounts of label in glutamate and glutamine. Cossins and Beevers (1963) found that ethanol was converted to organic acids, amino acids, lipids, and sugars in a wide variety of plant tissues. In pea cotyledons the major products labelled were fats as compared to sugars in the endosperms of castor beans.

Plants vary widely in their ability to tolerate anaerobiosis and ethanol toxicity. There are several adaptive strategies utilized by plants to avoid the phytotoxic effects of anoxia or hypoxia (Hochachka and Somero, 1973). The avoidance strategy involves the loss of ethanol from tissues to prevent damage. Ethanol produced under anoxic conditions is volatile and readily diffuses out of plant tissues (Barclay and Crawford, 1982). In plants where roots are submerged there is a diffusion of oxygen to and of ethanol away from the roots (Crawford and Zochowski, 1984). Grineva (1963) found that corn and sunflower seedlings excrete ethanol from their root systems during periods of anoxia. Rice seedlings have been shown to excrete up to 98% of the ethanol they produce into the imbibing medium (Bertani et al., 1980) and Echinochloa crus-gali seeds excrete up to 85% of the ethanol they produce (Rumpho and Kennedy, 1981).

The compensatory strategy is illustrated by the Pasteur effect. Glycolysis and fermentation are accelerated to maintain ATP production and reducing potential in the absence of aerobic respiration.

The metabolic switch or exploitative strategy has been proposed for flooding tolerant species which in some cases have been found to produce lactate or malate instead of ethanol under hypoxic conditions (Crawford and Tyler, 1969). Crawford (1977) theorized that seeds tolerant to anoxia had low anaerobic respiration rates, a minimization of the Pasteur effect and produced lactate as a less toxic metabolic endproduct rather than ethanol. Joly and Crawford (1982) found that wetland species reached a plateau of ethanol accumulation which remained constant during continued anoxia. These species resisted anoxic injury by maintaining a steady state in which ethanol loss equalled production. Species which are intolerant of flooding exhibit increased rates of glycolysis and ADH induction and may be excluded from wetland habitats because they produce toxic levels of ethanol (Crawford, 1966 and Crawford and Tyler, 1969). Other research, however, has showed that the major endproduct of anaerobic respiration is most usually ethanol (John and Greenway, 1976 and Keeley, 1978) and that rice (Taylor, 1942) and barnyard grass (Rumpho and Kennedy, 1981), two species tolerant of anoxia, exhibit strong Pasteur effects.

Temperature affects respiration rates and so, can influence ethanol production (Cannon, 1925). Barclay and Crawford (1981) found an interaction between temperature during anoxia and seedling survival. They found that seedlings were more tolerant of anoxia at 5 degrees C than at 25 degrees C. Ethanol concentration increased with duration of anoxia and with temperature.

Various environmental conditions can influence the intensity and duration of the metabolic production of ethanol including flooding and soaking injury and ice encasement in cereal (Andrews and Pomeroy, 1977). These conditions cause anoxia by effectively limiting oxygen diffusion to germinating seeds and seedlings. Excess water limits oxygen availability because of the relatively low solubility of oxygen in water (Pollock, 1972). When seeds are planted in wet soils or when water logging occurs, anaerobiosis can cause "soaking injury" associated with anaerobic production of ethanol (Crawford, 1977, and Rowland and Gusta, 1977).

It has been suggested by several authors (Spragg and Yemm, 1959, Ohmura and Howell, 1962, Cameron and Cossins, 1967, Leblova et al., 1969, and Pesis and Ng, 1986) that germinating seeds undergo a period of natural anaerobiosis prior to radical protrusion. Kolloffel (1967) outlines the respiration of germinating pea seeds dividing it into four phases. In phase II, a lag in oxygen uptake occurs, along

with an increase in RQ indicating increased glycolytic activity. It was suggested that the seed coat restricts oxygen supply before radical protrusion. At the onset of phase III radical protrusion is accompanied by a drop in RQ and an increase in oxygen uptake. This alone, however, does not explain the lag phase as seed coat removal and increase of oxygen level fail to eliminate the phase. Solomos et al. (1972) found an RQ of greater than unity in imbibing pea cotyledon slices which indicates that resistance to oxygen diffusion by the seed coat alone cannot explain ethanol production. It was concluded that anaerobic respiration may be due to the presence of underdeveloped mitochondrial structure. The mitochondrial cristae in air-dry seeds have been shown to be poorly developed (Abdul-Baki and Baker, 1973). The biochemical integrity of the mitochondria as measured by respiratory control ratio (RCR) and phosphorylative efficiency increases in early imbibition (Kolloffel and Sluys, 1970). Bain and Mercer (1966) found that oxygen uptake increased as mitochondrial development progressed during germination and predicted that glycolytic enzymes which were cytoplasmic would become activated before oxidative enzymes associated with the mitochondrial cristae. De la Fuente-Burguillo and Nicolas (1974) found that the cotyledons of Cicer arietinum contain the enzymes necessary for the conversion of glucose to pyruvate and pyruvate to

ethanol. They found that during the first 24 hours of germination there was a relatively greater amount of glycolytic enzyme activity relative to citric acid cycle enzyme activity. Morohashi and Shimokoriyama (1975b) found that activation of glycolysis precedes that of the citric acid cycle in peas. It is possible that pyruvate production rates exceed the capacity of the underdeveloped citric acid cycle to further metabolize the amount of pyruvate present. Davies et al. (1974) state that pyruvate buildup can induce ethanol production. De la Fuente-Burguillo and Nicolas (1974) found increased activity of glycolytic enzymes and ADH during the period before radical protrusion in Cicer arietinum. Kolloffel (1968), found sustained ADH activity up until the time of radical protrusion at which time it dropped to low levels.

Increased ethanol production has also been associated with vigor decline in both natural and artificial aging. Deteriorated seeds are more sensitive to anoxia than vigorous seeds (Ellerton and Perry, 1983). Many investigators have reported solute leakage from low vigor or aged seeds indicative of membrane damage (Koostra and Harrington, 1969, Abdul-Baki, 1980, and Woodstock and Grabe, 1967). They have suggested that the decline in vigor and viability can be attributed to damage to mitochondrial membranes. This can lead to a decrease in aerobic respiration and a resulting

imbalance between glycolysis and the citric acid cycle (Woodstock and Taylorson, 1981a, and Pesis and Ng, 1984a). Woodstock and Taylorson (1981a) found that after eight hours of soaking, low vigor soybean seeds produced more ethanol for a longer period of time than high vigor seeds. Anaerobic conditions and treatment with sodium azide induced production of ethanol at rates similar to low vigor seeds. Stewart and Beweley (1980) found that peroxidation of lipids in the plasmalemma of low vigor seeds may lead to membrane deterioration which causes loss of solute control and affects membrane associated enzymes. The effect noted was the buildup of the products of glycolysis and production of ethanol. Morohashi and Shimokoriyama (1975b) found that the mitochondria of dry seeds were inactive, and upon wetting the mitochondra of vigorous seeds became rapidly functional. Pradet (1982) and Abdul-Baki (1980) found that, in contrast, the mitochondra of aged seeds became functional more slowly. Pesis and Ng (1986) found that artificial aging treatments damaged the aerobic respiratory pathway and that the longer the duration of the aging treatment, the more ethanol was produced. Harmon et al. (1982) found that the production of volatile aldehyde compounds, including ethanol, increased with poor soybean and pea seed quality and was correlated with field emergence. Gorecki et al. (1985) found that unaged seeds with the highest vigor released the least amount

of volatiles (primarily ethanol and acetaldehyde) and that unaged pea seeds produced ethanol up to 12 hours into germination at which time production decreased. In aged seeds however, ethanol continued to increase with imbibition time. They felt that mitocondrial dysfunction persisited for a longer period of time as vigor declined. The longer the establishment of mitochondrial integrity and function is delayed, the more pyruvate builds up and is channeled into ethanol production. These authors found a strong negative correlation between ethanol and acetaldehyde production and seed vigor. Pesis and Ng (1986) found that the number of abnormal seedlings increased and germination percentage decreased as production of ethanol increased in imbibing seeds. A decline in oxygen uptake in aged seeds has also been attributed to membrane damage. This, along with the higher RQ reported by Pesis and Ng may indicate anaerobic respiration. Gorecki et al. (1985) felt that ethanol production may provide a good measure of the physiological state of the seed. The concentration of ethanol in seeds may be indicative of the time and level of anaerobic respiration, but is not the primary cause of loss of viability (Davies, 1980).

MATERIALS AND METHODS

Corsoy 79 soybean seed, harvested in 1985, was obtained from the Committee for Agricultural Development, Ames, Iowa. Seed was sized and any seed showing visible cracks was discarded prior to use. Two different artificial aging methods were applied to both surface sterilized and nonsurface sterilized seed. Surface sterilized seed were exposed to 0.5% hypochlorite for 30 seconds and then washed under running tap water for 30 seconds.

The first aging method consisted of a relative humidity of greater than 90% and a temperature of 42 degrees C for durations of 1 to 3 days (AOSA, 1983). Moisture content of aged seed was 18.6%, 26.4%, and 30.4% after 1,2, and 3 days of aging, respectively.

The alternate method used was a modification of the technique outlined by Edje and Burris (1970). The moisture content of the soybean seed was adjusted to 12% by placing seed on screens in germination carts containing trays with moistened Kimpak (Kimberly-Clark Corp.). Seed samples at the 12% moisture level were sealed in Ball canning jars and placed in a 40 degree C oven for durations of up to 23 days. Loss of moisture during aging averaged 0.3%. Seed aged by both aging methods were held at 10 degrees C and 50% relative humidity until used for further testing. After the aging

treatments, standard germination and conductivity tests were conducted.

Seed were imbibed at ambient room conditions (21 degrees C and 30% relative humidity) with glass beads 1 millimeter in diameter as the substrate (Thomas Scientific). Twenty-five milliliters of deionized water were added to crystallization dishes, 10 seeds were placed in each dish and then covered with a moist filter paper and an inverted petri dish to prevent drying. Seeds were imbibed for periods of 2, 4, 8, 12, 24, and 34 hours with 3 replicates of 10 seeds at each time. Preliminary imbibition trials indicated that radicle protrusion, the criterion for physiological germination, occurred at 34 hours. At each imbibition interval, the seeds were rinsed, blotted dry, and placed in a 50 ml Erlenmeyer flask which was then sealed with a septum. After one half hour, 1 cc of headspace gas was removed from each flask and injected into a Varian 2700 gas chromatograph with flame ionization detector. A Carbopack (Supelco) column was used and the retention time for ethanol was 1 minute. Operating temperatures were as follows: injector port, 80 degrees C, column, 85 degrees C, and flame ionization detector, 110 degrees C. Standard solutions of ethanol (Aldrich) were used to identify the retention time of ethanol and to construct a standard curve to convert peak height to millimoles ethanol. A Porapak Q column (Supelco) was used to verify peak

identification. After peak measurement, seeds were weighed, dried for 24 hours at 103 degrees C and moisture calculated on a fresh weight basis.

RESULTS AND DISCUSSION

Imbibition

Water uptake is affected by temperature, imbibition substrate, the amount of free water, seed size, and the presence of hard seeds (Harper and Benton, 1966). Much of the data on water uptake has been collected after immersing seeds in water. Such methodology can result in imbibition injury and the development of anoxic conditions which can adversely affect subsequent germinative processes (Rowland and Gusta, 1977, Woodstock and Taylorsen, 1981a, and Anderson and Baker, 1983). These conditions could make it difficult to distinguish betwen production of ethanol due to vigor decline and production resulting from membrane damage sustained during rapid water uptake, or anoxia. An imbibition methodology was needed that would allow germination to proceed in a timely manner, but avoid the negative effects of immersion. Germination on Kimpak is utilized by the Iowa State University Seed Laboratory, Ames, Iowa, and thought to provide a reasonable imbibition rate when wet according to the International Rules for Seed Testing (ISTA, 1966) guidelines of 60% of water holding capacity. Glass beads provided a similar uptake pattern with less among replicate variation.

A typical phasic course of water uptake was observed (Fig. 1) with rapid water uptake followed by a period of much

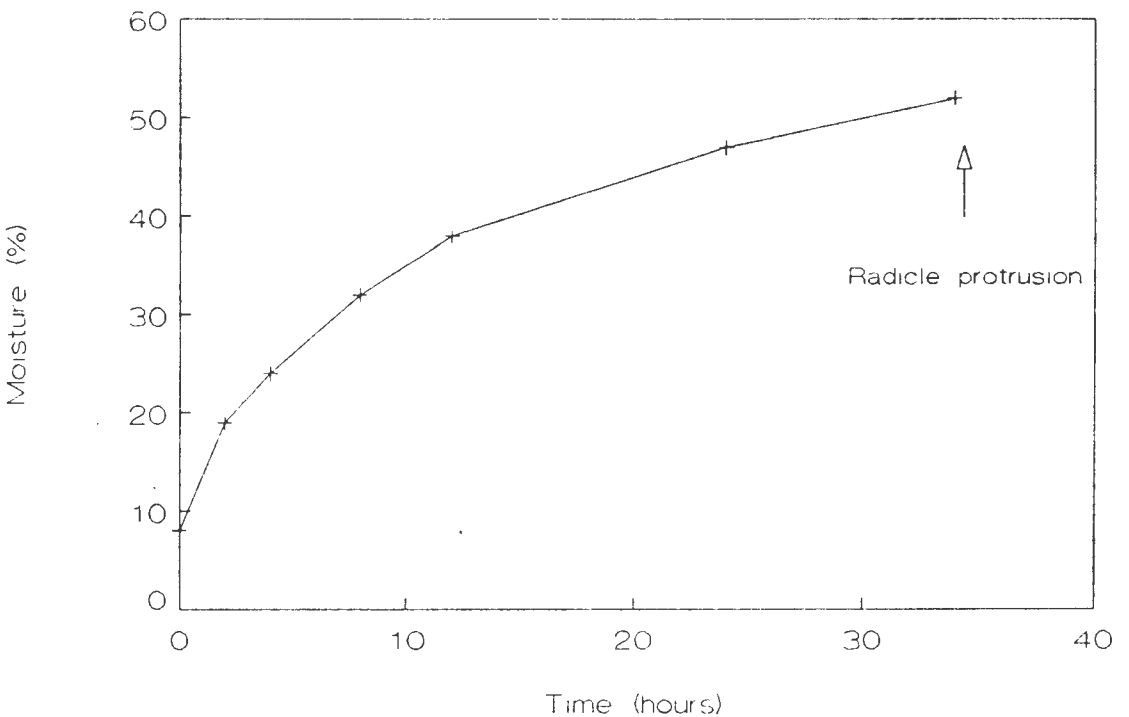

Figure 1. Water uptake by Corsoy 79 soybeans

slower uptake from 12 hours to radicle protrusion. This period roughly corresponds to the lag phase as described by Beweley and Black (1983) and a reduced water potential gradient between the seed and imbibition medium contributes to the much slower uptake rate. The nature of the lag period is species, seed, and temperature dependant (Hegarty, 1978). Berrie (1984) suggests that different parts of an individual seed pass through the different imbibition phases at different rates so the net imbibition curve does not show a distinct lag phase. The third phase of water uptake was not observed because imbibition was stopped at radicle protrusion and the increase in uptake associated with Phase III is thought to be due to axis growth and generation of vacuolar sap (Berrie, 1984). All seeds began imbibition at approximately 8.5% moisture. Vigor should have no effect on imbibition rate as reported by Woodstock and Tao (1981) and Cseresnyes and Vorenvenci (1984). In control (unaged) seed, time of imbibition was the only factor which significantly increased seed moisture content. However, in both aging methods used, aged seed had a significantly higher moisture content than unaged seed at the same imbibition time (Tables 2-4). Lack of oxygen uptake data makes it impossible to estimate the respiratory rates at this point, but it is possible that, as imbibition progressed, there was some active uptake occurring which may be affected by respiratory

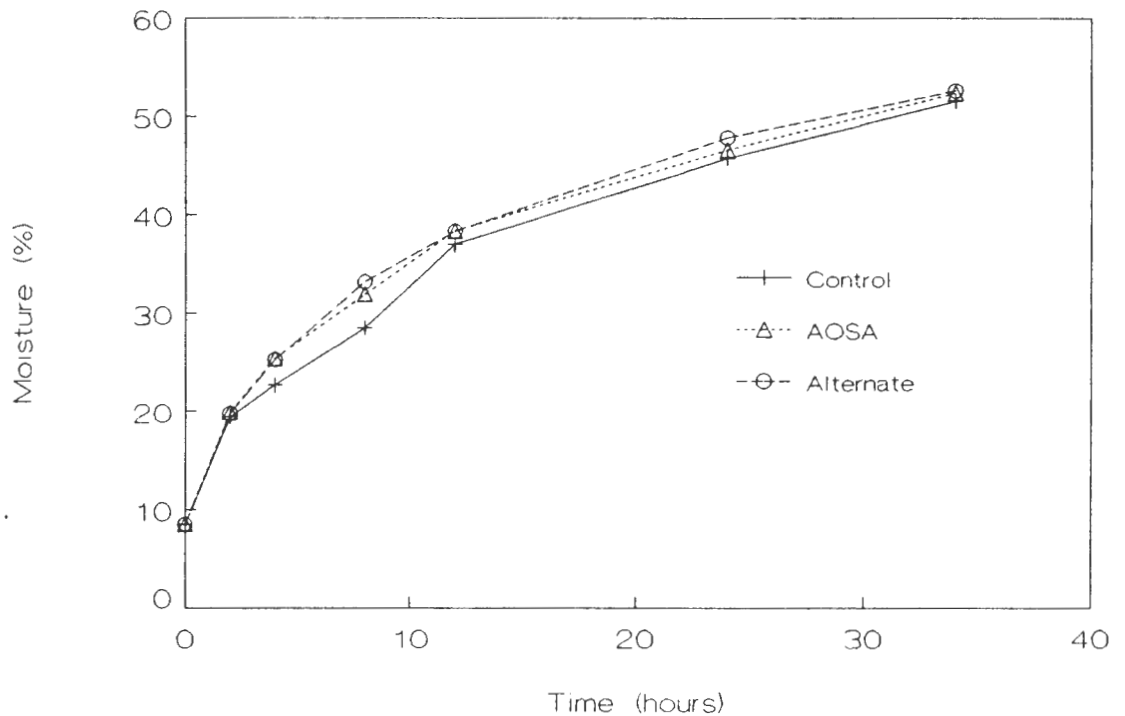

Figure 2. Water uptake by Corsoy 79 soybeans as affected by aging treatment

rate (Morohashi and Shimokoriyama, 1972b). The time of greatest difference in moisture between aged and unaged seed occurred during imbibition times of 2 to 12 hours (Fig. 2) and moisture contents of 20 to 38%. In both aging methods seed moisture content is elevated and seed is then air dried before further imbibition. When seeds are allowed to imbibe to the second phase of water uptake, dried and later introduced to water again, they imbibe more rapidly to the point of radicle emergence (Beweley and Black, 1983). Aging treatments may also affect seed coat integrity, leading to more rapid water uptake.

Artificial Aging and Ethanol Production

Temperature and moisture are two variables which affect seed deterioration. It is not unexpected then, that the AOSA method of aging, which utilizes both variables causes a much more rapid decrease in viability than does the alternate aging method which utilizes only elevated temperature. The ability to produce normal seedlings was lost at a fairly constant, rapid rate when seed were exposed to both high temperature and humidity. A greater proportion of the remaining seed was categorized as abnormal than dead. In contrast, the ability to produce normal seedlings was lost more slowly by aging with the alternate method. It took 17 days to reach the same decline in viability that was reached in 3 days using the AOSA method (Table 1) and at this

Table 1. Average germination and conductivity values for aged soybeans

Treatment	Normal (%)	Abnormal (%)	Dead (%)	Conductivity (microamps)
Control	98	2	0	85.86
Control (SS)	97	3	0	87.95
AOSA 1	78	18	4	103.88
AOSA 1 (SS)	82	16	2	105.18
AOSA 2	57	33	10	116.37
AOSA 2 (SS)	61	27	11	105.68
AOSA 3	35	44	21	168.68
AOSA 3 (SS)	42	40	18	178.20
Alt. 1	98	2	0	86.29
Alt. 1 (SS)	97	3	0	83.87
Alt. 2	96	4	0	97.42
Alt. 2 (SS)	96	3	1	87.07
Alt. 3	95	4	1	88.41
Alt. 3 (SS)	94	6	0	91.00
Alt. 5	92	6	2	86.80
Alt. 5 (SS)	94	6	0	83.80
Alt. 7	90	8	2	87.30
Alt. 7 (SS)	91	8	1	92.99
Alt. 10	85	10	5	96.96
Alt. 10 SS)	82	12	6	89.69
Alt. 14	75	13	12	92.65
Alt. 14 (SS)	76	10	14	107.49
Alt. 17	37	18	45	165.95
Alt. 17 (SS)	42	20	38	221.29
Alt. 20	9	13	78	214.87
Alt. 20 (SS)	13	8	79	241.72
Alt. 23	0	0	100	232.60
Alt. 23 (SS)	1	2	97	234.97

viability level the alternate method produced twice as many dead seeds.

Sampling of headspace above imbibing seeds gives a good qualitative estimate of the amount of ethanol produced (Barry Martin, Pioneer Hy-Bred, International, personal communication) although the proportion of ethanol excreted into the imbibing medium varies with species and temperature. Bertani et al. (1980) and Rumpho and Kennedy (1981) found that most of the ethanol produced by rice and Echinochloa crus-galli was excreted while Crawford and Zochowski (1984) found that the headspace above imbibing pea seeds contained only about 5% of the total ethanol produced. There is rapid equilibration between imbibition medium and air; less than 15 minutes was required for equilibration between standard solutions of ethanol and headspace.

In unaged controls, ethanol production peaked at 4 hours, or at a moisture content of about 24%, and returned to a low level by 8 hours of imbibition (Fig. 3). As vigor and viability declined, the amount of ethanol evolved increased, peak ethanol evolution occurred later in imbibition, and occurred at a higher moisture content (Table 8). Seed aged using the AOSA method evolved peak amounts of ethanol at 8 hours regardless of the aging interval. Seeds imbibed after all aging intervals had elevated ethanol production at both the first and last measurement of testing than unaged

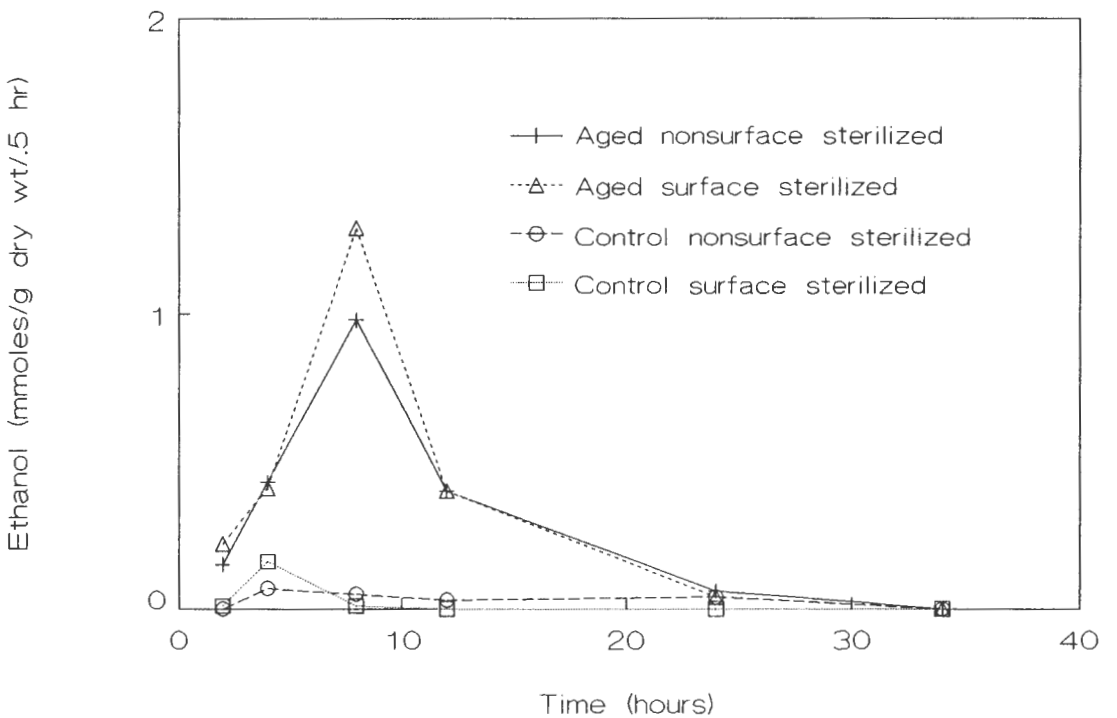

Figure 3. Ethanol evolution rates versus imbibition time for unaged controls and seed aged 1 day using the AOSA aging method

controls (Fig. 3-5). After 3 days of aging, recovery was impaired and ethanol production remained at a high constant level in surface sterilized seed and declined slowly in nonsurface sterilized seed (Fig. 5). Seeds aged using the alternate aging method showed similar patterns of ethanol evolution (Fig. 6-8), that is, an increase from a basal level to a peak and then a decline. Increased ethanol evolution coincided with a decline in germination percent after 10 days of aging and peak evolution occurred at 8 hours. By 17 days of aging, peak ethanol evolution was delayed until 24 hours, and again, there was a failure of recovery to low levels. Some ethanol is produced normally by germinating seeds due to the lag in activation of membrane associated enzymes of the citric acid cycle compared to glycolytic enzymes (Morohashi and Shimokoriyama, 1975b). Ethanol production appears to be more dependant on membrane integrity and establishment of the citric acid cycle than of the hydration of glycolytic enzymes. The mitochondria of vigorous seeds become functional rapidly with hydration while the activity of mitochondrial enzymes is retarded temporally in aged seeds (Pradet, 1982 and Abdul-Baki, 1980). Accelerated aging damages the aerobic respiratory pathway and the longer the duration of aging treatment, the more ethanol is produced (Pesis and Ng, 1986). This is the observed result in both aging methods; as viability decreases, more ethanol is

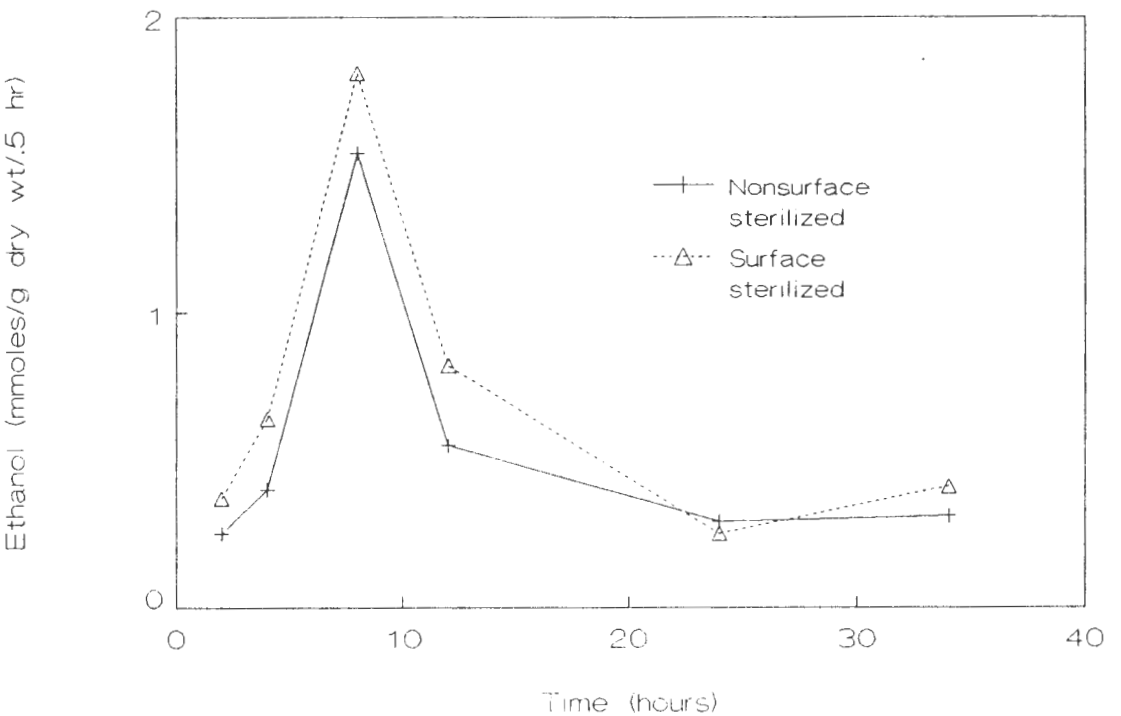

Figure 4. Ethanol evolution rates versus imbibition time for seed aged 2 days using the AOSA aging method

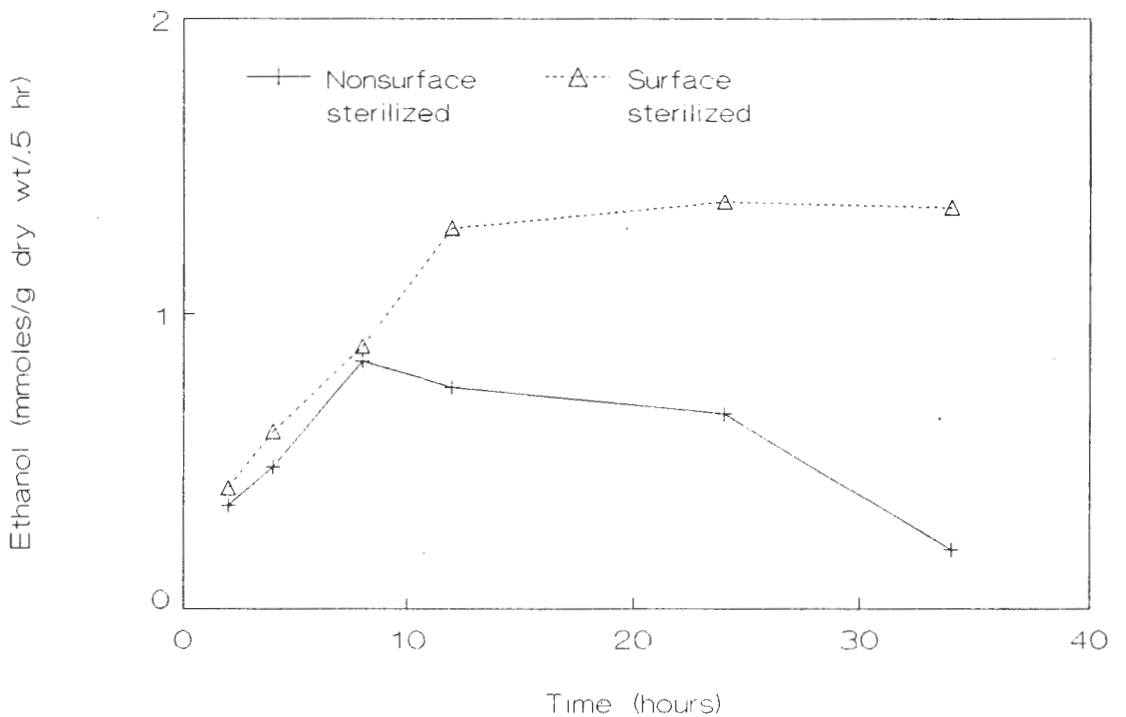

Figure 5. Ethanol evolution rates versus imbibition time for seed aged 3 days using the AOSA aging method

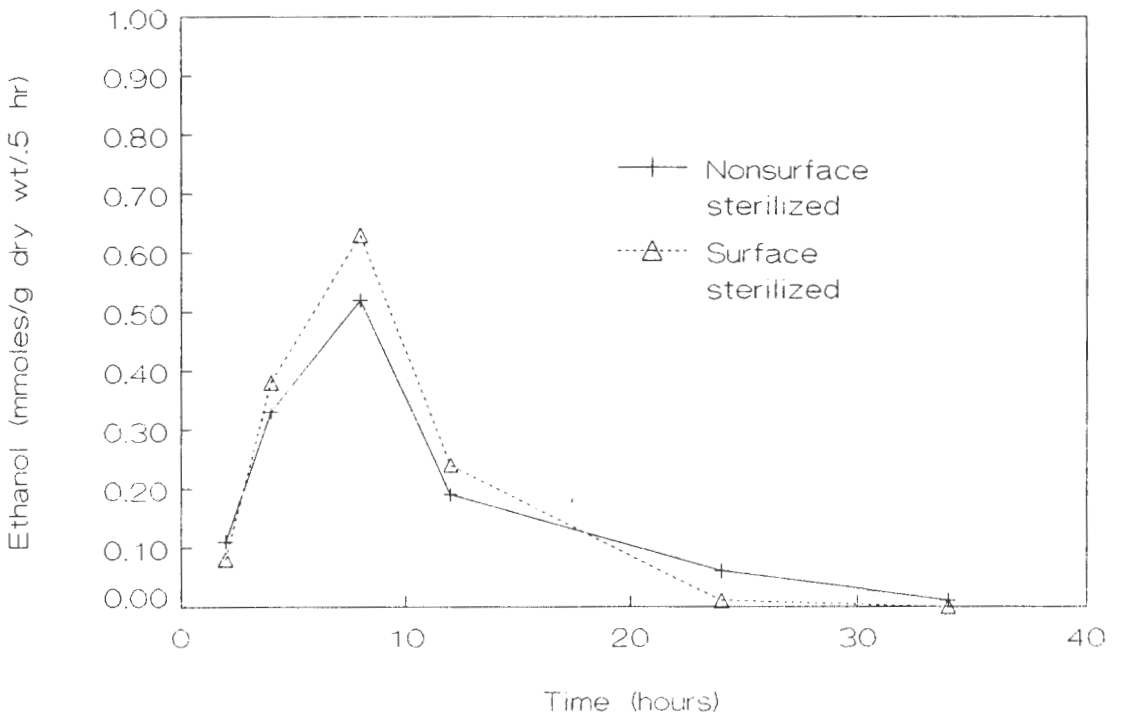

Figure 6. Ethanol evolution rates versus imbibition time for seed aged 7 days using the alternate aging method

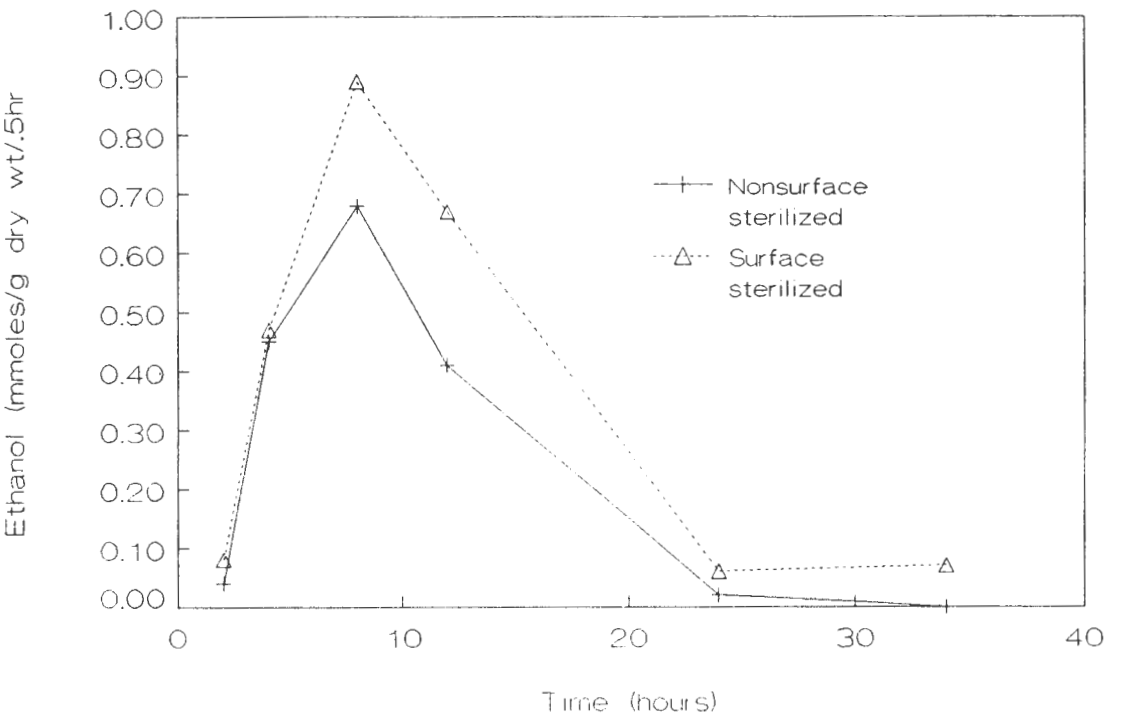

Figure 7. Ethanol evolution rates versus imbibition time for seed aged 14 days using the alternate aging method

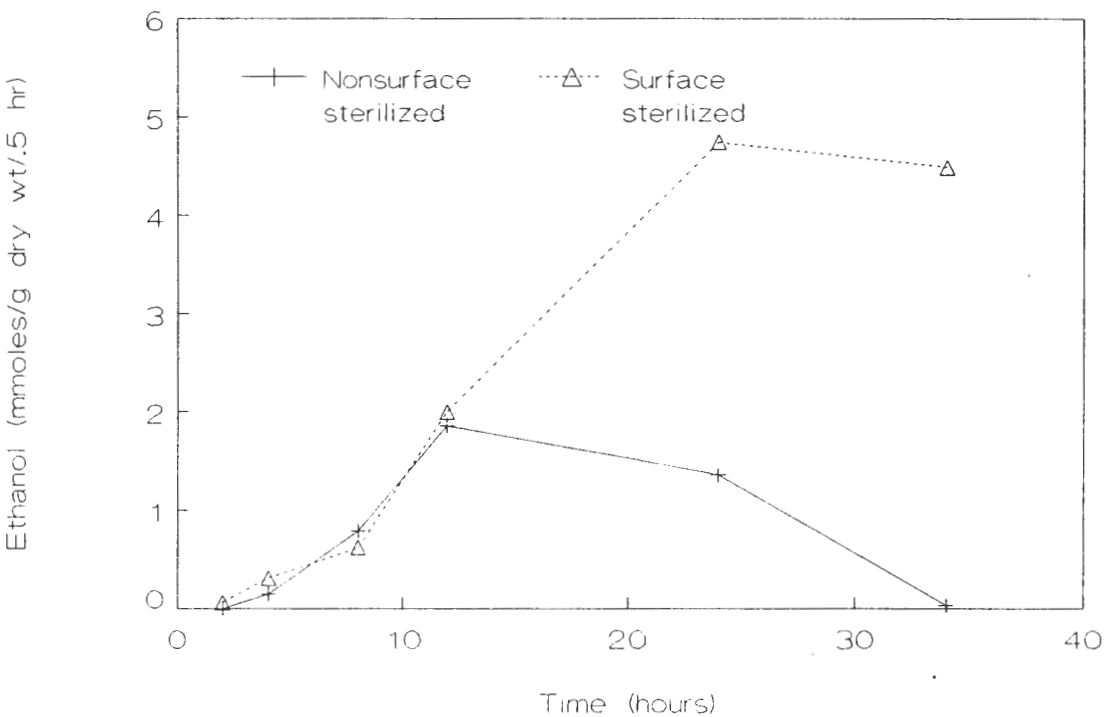

Figure 8. Ethanol evolution rates versus imbibition time for seed aged 17 days using the alternate aging method

produced. An increase in the duration of aging results in a decrease in germination, an increase in abnormal seedlings, and in conductivity (Table 1), indicating increased permeability of membranes. The increased time to peak ethanol production may be due to the increased time necesary to attain membrane integrity. During the lag between activation of glycolytic enzymes and citric acid cycle enzymes, the buildup in pyruvate levels would lead to production of ethanol (Beevers, 1961). ATP depletion could lead to high levels of ADP, an allosteric activator of pyruvate decarboxylase, which is thought to control ethanol production (John and Greenway, 1976 and Chang et al., 1982).

Spragg and Yemm (1959), Ohmura and Howell (1962), Cameron and Cossins (1967), Leblova et al. (1969), and Pesis and Ng (1986) found increased production of ethanol up until radicle protrusion in the germination of various species and suggested that the seed coat may restrict oxygen supply, causing anoxic production of ethanol. Increased evolution of ethanol up until radicle protrusion was not observed and the temporal shift in peak production noted would be more supportive of delayed membrane organization.

With continued decline in viability, recovery from production of ethanol slowed or failed completely. Stotzky and Schenk (1976) found that production of volatiles increased over a longer period of time as germinability of

seeds declined, which was consistent with observed results. Higher average levels of ethanol may indicate either a high peak level of ethanol production or a longer period of elevated production. Crawford (1977) proposed that as more ethanol was produced, enough accumulated to have toxic effects on cell membranes, increasing the already existing imbalance between glycolysis and the citric acid cycle. The extent of ethanol induced damage is dependant on the rate of ethanol production, the length of the time period before accumulated ethanol is metabolized, and the extent that the produced ethanol is metabolized (Chang et al., 1982). All of these variables would tend to create elevated internal ethanol concentrations. Ethanol toxicity is not thought to be a primary cause of vigor loss but may play a secondary role. When ethanol accumulates in the cells it may exert a fluidizing effect on membranes, further damaging the already impaired citric acid cycle apparatus. If there is a dysfunction in the citric acid cycle, the metabolism of ethanol as outlined by Cameron and Cossins (1967) may be impaired which would result in a buildup of ethanol. Substrate induction of ADH could also lead to increased ethanol production. Barclay and Crawford (1981) found that an internal ethanol concentration of 60 mM caused anoxic injury in pea seedlings. If a minimum of 5% of this amount of ethanol is assumed to diffuse out of the seed into the

surrounding headspace (Crawford and Zochowski, 1984), 3mM would be required to indicate internal concentrations necessary to produce anoxic injury. Where there is a failure to recover from high ethanol production after extended aging, this amount is attained or exceeded and ethanol induced damage may have occurred.

Ethanol production resulting from the two aging methods was compared at similar viability levels. In comparing one day of aging by the AOSA method with 10 days by the alternate method (Fig. 9), the AOSA method gave a sharper, higher peak and also a higher average production than did the alternate method, however, comparing 3 days aging by the AOSA method with 17 days by the alternate method (Fig. 10), the trend was reversed. After 17 days of aging, the AOSA method had a greater number of abnormal seedlings than did the alternate method. This observation leads to the question of the contribution of abnormal and "dead" seeds to the amount of ethanol measured. According to Harrington (1973), a nonviable seed is one in which there is no protrusion of plant parts when the seed is placed under conditions ideal for germination. Vancura and Stotzky (1976) state that volatile aldehyde compounds including methanol, ethanol, acetaldehyde, propionaldehyde, and acetone are products of metabolism and therefore not evolved from dead seeds. This may mean that the viable seeds, including seeds which produce

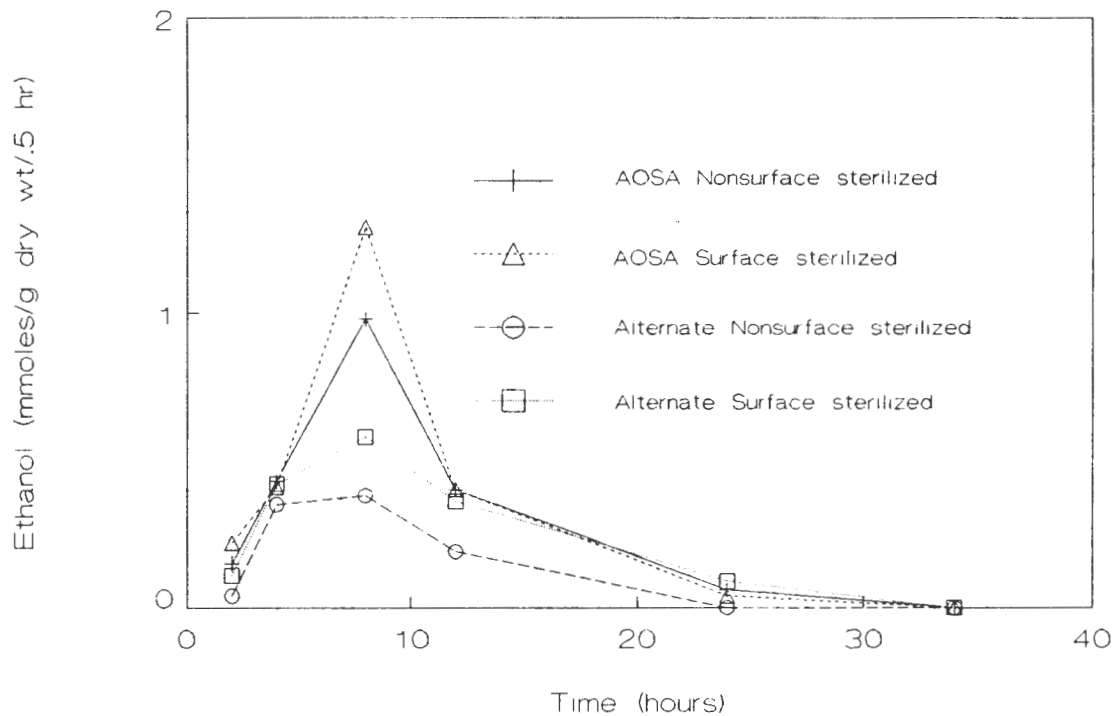

Figure 9. Comparison of ethanol evolution between methods at similar levels of viability; 1 day using the AOSA aging method and 10 days using the alternate aging method

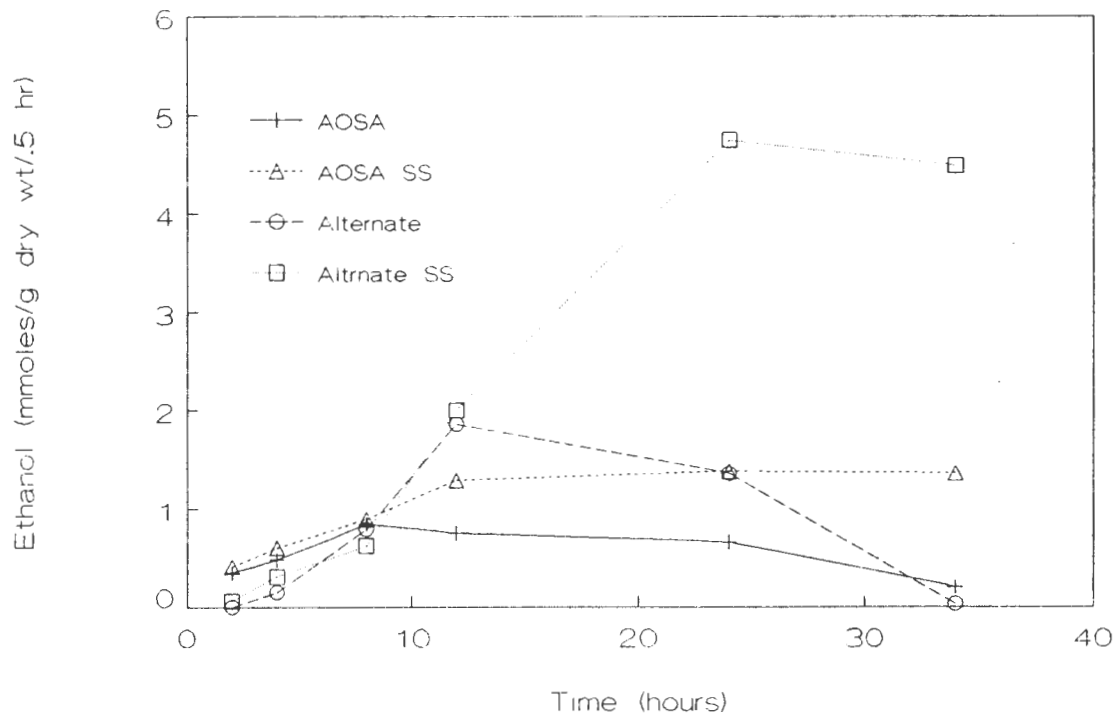

Figure 10. Comparison of ethanol evolution between methods at similar levels of viability; 3 days using the AOSA aging method and 17 days using the alternate aging method

both normal and abnormal seedlings, aged by he AOSA method do not produce as much ethanol as do those of the alternate method, suggesting the possible involvement of two different mechanisms of deterioration. Alternatively, a possible contribution from nonviable seeds to ethanol measured cannot be overlooked. Harrington (1973) found enzyme activity in nonviable seeds and McLeod (1952) found a lag between loss of viability and loss of dehydrogenase activity. A lag between loss of viability and loss of enzyme activity in seeds classified as dead may explain the high production of ethanol from aged seeds with a very low percentage of viable seeds. The presence of microorganisms on seed may also contribute to the observed results. The AOSA method of aging is highly conducive to the growth of storage fungi (Anderson and Baker, 1983), while at a constant 12% moisture content, the activity should be minimal. When viability of seeds declines to comparably low levels after 3 days of aging by the AOSA method and 17 days of aging by the alternate method, less ethanol is evolved by seeds aged using the AOSA method. It is possible that utilization of ethanol by microorganisms may decrease measured amounts of ethanol.

There was a high negative correlation between average ethanol production in 34 hours and number of normal seedlings over both aging methods and sterilization treatments (Table 9). Average ethanol production and conductivity were also

highly correlated. There was no significant correlation between ethanol production and the number of abnormal seedlings when both methods were combined in the analyses, however, when analyzed separately, there was a significant positive correlation between ethanol production and abnormal seedlings in the AOSA method of aging (Tables 10-11). Average ethanol production over 34 hours was better correlated with germination than was peak ethanol evolution. In the alternate aging method here was no significant correlation between ethanol production and abnormal seedlings. Correlations using peak or average production were not significantly different.

Surface Sterilization

Significantly greater amounts of ethanol were measured in the headspace above surface sterilized when compared to seed which had not been surface sterilized. An interaction between surface sterilization and days aged was also observed (Tables 4-6). Harmon et al. (1982) and Gorecki et al. (1985) found that seedborne microorganisms may decrease the measured amount of volatiles evolved by germinating seeds by up to 80%. Other researchers are concerned that infection may increase the amount of volatiles measured due to production by the organisms themselves (Avadhani et al., 1978). Woodstock and Combs (1965), however, found that respiration of microorganisms was negligible when compared to that of the

seeds. The AOSA method of aging is highly conducive to the growth of storage fungi (Anderson and Baker, 1983) and the presence of microorganisms on the seed surface may contribute to the decrease in ethanol evolved by seed which was not surface sterilized. However, surface sterilized seed aged by the alternate method also produced more ethanol then nonsurface sterilized seed and 12% moisture is generally considered to be low for growth of storeage fungi (Christiansen, 1972).

Although surface sterilization using sodium hypochlorite is a common laboratory practice for controlling saprophytic fungi in seed lots, there has been little research conducted to determine the physiological effects of such treatment. The relatively mild exposure to hypochlorite in this study caused a noticeable wrinkling of soybean seed coats and after 30 seconds of treatment and rinsing seed, moisture content increased an average of 2%. Surface sterilized seed had a significantly higher moisture content at the same imbibition times than did nonsurface sterilized seed (Table 12). A general trend of increasing conductivity relative to nonsurface sterilized seed was noted in seed which had been surface sterilized (Table 1), however, the increase was not statistically significant (data not shown). Abdul-Baki (1974) found that residual hypochlorite from surface sterilization was reactive with amino acids and caused high

production of carbon dioxide and low uptake of amino acids. While this illustrates the potential for a physiological effect on water uptake and ethanol production in imbibing seeds due to treatment with hypochlorite, specific effects are unknown.

Conclusion

In conclusion, the metabolic production of ethanol increases with a decrease in vigor. Production increases from a basal level to a peak and then declines. This point in time may correspond to the period when the citric acid cycle becomes functional. With declining vigor, this time increases until there is no decline in ethanol evolution which may indicate that toxic levels of ethanol have accumulated.

This study provides no conclusive evidence of differences in aging mechanisms between the two methods used for artificial aging. The long periods of high temperature at a constant 12% moisture may cause different, or at least much slower internal changes in the seed than do the shorter periods of aging at high temperature and high moisture content. Differences in ethanol measurements between surface sterilized and nonsurface sterilized seed should not be attributed entirely to the presence of microflora on the seed without further research to determine the physiological and biochemical effects of hypochlorite on cell constituents.

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APPENDIX

Table 2. Analysis of variance for unaged controls: Dependent variable, moisture

Source of variation	d.f.	Mean square	F value
Replicate	2	0.988	0.30
Sterilization	1	8,381	2.52
Imbibition	5	991.500	298.51**
Ster*Imb	5	1.278	0.38
Error	22		

**Significant at the .01 level.

Table 3. Analysis of variance for AOSA method of aging: Dependent variable, moisture

Source of variation	d.f.	Mean square	F value
Replicate	2	0.343	0.28
Sterilization	1	9.464	7.76**
Imbibition	5	2786.896	2285.62**
Ster*Imb	5	1.405	1.15
Day	2	12.053	9.88**
Ster*Day	2	1.082	0.89
Imb*Day	10	3.109	2.55
Ster*Imb*Day	10	0.492	0.40
Error	70		

**Significant at the .01 level.

Table 4. Analysis of variance for alternate aging method: Dependent variable, moisture

Source of variation	d.f.	Mean square	F value
Replicate	2	0.843	0.89
Sterilization	1	44.114	46.65**
Imbibition	5	9688.969	10245.81**
Ster*Imb	5	2.317	2.45
Day	9	21.180	22.40**
Ster*Day	9	1.796	1.90**
Imb*Day	45	7.011	7.41**
Ster*Imb*Day	45	1.405	1.49
Error	238		

**Significant at the .01 level.

Table 5. Analysis of variance for unaged controls: Dependent variable, ethanol

Source of variation	d.f.	Mean square	F value
Replicate	2	0.0004	0.64
Sterilization	1	0.0001	0.13
Imbibition	5	0.0162	26.80**
Ster*Imb	5	0.0041	6.82**
Error	22		

**Significant at the .01 level.

Table 6. Analysis of variance for AOSA method of aging: Dependent variable, ethanol

Source of variation	d.f.	Mean square	F value
Replicate	2	0.0101	0.18
Sterilization	1	1.2770	22.45**
Imbibition	5	2.1077	37.05**
Ster*Imb	5	0.0687	1.21
Day	2	1.5476	27.20**
Ster*Day	2	0.3486	6.13**
Imb*Day	10	0.5323	9.36**
Ster*Imb*Day	10	0.1418	2.49**
Error	70		

**Significant at the .01 level.

Table 7. Analysis of variance for alternate method of aging: Dependent variable, ethanol

Source of variation	d.f.	Mean square	F value
Replicate	2	0.1848	2.39
Sterilization	1	0.4957	6.40**
Imbibition	5	15.9250	205.77**
Ster*Imb	5	0.1386	1.79
Day	9	24.0501	310.76**
Ster*Day	9	2.5655	33.15**
Imb*Day	45	7.3392	94.83**
Ster*Imb*Day	45	1.2100	15.63**
Error	238		

**Significant at the .01 level.

Table 8. Average and peak ethanol production (mmoles/g dry wt) for aged soybeans

Treatment	Average	Peak	Imb. Time (hrs)	Moisture (%)
Control	0.0272	0.0736	4	24
Control (SS)	0.0242	0.1667	4	24
AOSA 1	0.3331	0.9858	8	32
AOSA 1 (SS)	0.3899	1.2898	8	32
AOSA 2	0.5580	1.5480	8	32
AOSA 2 (SS)	0.7166	1.8075	8	32
AOSA 3	0.5491	0.8489	8	32
AOSA 3 (SS)	0.9861	1.3787	8	32
Alt. 1	0.0928	0.2287	4	24
Alt. 1 (SS)	0.0402	0.1457	4	24
Alt. 2	0.1069	0.3121	4	24
Alt. 2 (SS)	0.0842	0.3139	4	24
Alt. 3	0.1090	0.2884	4	24
Alt. 3 (SS)	0.2069	0.5764	4	24
Alt. 5	0.0538	0.2067	4	24
Alt. 5 (SS)	0.1188	0.4689	4	24
Alt. 7	0.2031	0.5165	8	32
Alt. 7 (SS)	0.2223	0.6333	8	32
Alt. 10	0.1549	0.3846	8	32
Alt. 10 (SS)	0.2539	0.5826	8	32
Alt. 14	0.2622	0.6801	8	32
Alt. 14 (SS)	0.3756	0.8882	8	32
Alt. 17	0.7492	1.8619	12	38
Alt. 17 (SS)	2.0399	4.9927	24	47
Alt. 20	2.2934	5.6814	24	47
Alt. 20 (SS)	2.3783	6.2307	34	52
Alt. 23	2.0798	7.3147	34	52
Alt. 23 (SS)	1.1271	3.5414	34	52

Table 9. Correlation coefficients for laboratory data using combined aging and sterilization treatments with ethanol evolution

Laboratory data	Ethanol evolved
Normal seedlings, %	-.882
Abnormal seedlings, %	.150
Dead seedlings, %	.868
Normal + Abnormal, %	-.868
Abnormal + Dead, %	.882
Conductivity, mamps	.928
Moisture, %	.093

Table 10. Correlation coefficients for AOSA and alternate methods of aging, surface sterilized and nonsurface sterilized with average ethanol evolution

	Ethanol evolved			
Laboratory data	AOSA	AOSA (SS)	Alt.	Alt. (SS)
Normal seedlings, %	-.920	-.993	-.960	-.855
Abnormal seedlings, %	.955	.997	.129	.511
Dead seedlings, %	.821	.962	.986	.793
Normal + abnormal, %	- 821.	-.962	-.986	-.793
Abnormal + dead, %	.920	.993	.960	.855

Table 11. Correlation coefficients for AOSA and alternate methods of aging, surface sterilized and nonsurface sterilized with peak ethanol evolution

	Ethanol evolved			
Laboratory data	AOSA	AOSA (SS)	Alt.	Alt. (SS)
Normal seedlings, %	-.617	-.247	-.952	-.896
Abnormal seedlings, %	.676	.763	.006	.437
Dead seedlings, %	.421	.648	.999	.844
Normal + Abnormal, %	-.421	-.648	-.999	-.844
Abnormal + Dead, %	.617	.247	.952	.896

Table 12. Moisture contents (%) during imbibition: comparison between surface sterilized and nonsurface sterilized seed

Imbibition Time	Control	Control (SS)	AOSA	AOSA (SS)	Alt.	Alt. (SS)
2	18.46	20.44	19.64	20.17	19.41	20.15
4	22.07	23.33	24.17	26.14	24.75	25.83
8	28.69	28.36	31.87	32.03	32.73	33.67
12	36.22	37.76	38.10	38.65	37.93	38.74
24	45.81	45.79	46.13	47.16	47.87	47.82
34	50.90	52.26	52.44	52.32	52.28	52.95